Die Hard - improving the physical quality of extruded fish feed pellets

Dethlefsen, Markus Wied

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

Die Hard

Improving the Physical Quality of Extruded Fish Feed Pellets

PhD thesis
Markus Wied Dethlefsen

Submitted
July 31, 2017

BioMar A/S
Process Innovation Team
Brande

DTU Food
National Food Institute
Technical University of Denmark

Supervisor
Bo Munk Jørgensen

Co-supervisors
Aberham Hailu Feyissa
Niels Harthøj Hjermitslev
Michael Engelbrecht Nielsen

PhD thesis

Markus Wied Dethlefsen

Die Hard

Improving the Physical Quality of Extruded Fish Feed Pellets

Front: Brande Station in the middle and BioMar's Danish factory in the top right-hand corner
Back: Municipal boundary in Brande

This project is funded by Innovation Fund Denmark (4135-00036)

Contents

Preface

When I back in November 2011 accepted a student job where I had to monitor the stability of BioMar fish feed, I was not fully aware of the consequences: Two years later, I defended a master's thesis about extrusion technology and fish feed production at BioMar, and additional four years later, I would hand in an industrial PhD thesis – again dealing with the aquaculture industry. When I arrived to BioMar in Brande to start the PhD project, my industrial supervisor, Niels Hjermitslev, passed on an internal technical handbook from 1993 to formally introduce me to the company and prepare me for the coming challenges. The preface of the handbook was very short and accurate, summarizing the field's essentials in one single sentence:

"Aquaculture has an almost magical ability to attract the innocent, lure them into its web, and ruin them."

After these prophetic words, I was fully motivated to get started.

Operators are very often blaming the raw materials if the physical quality of the extruded fish feed is poor. And the raw materials do have a finger in the pie. Changing from one ingredient to another or increasing the level of one can highly influence the physical pellet quality (PPQ). The pore structure is known to be very sensitive to recipe changes and is as well known to influence the overall product quality – especially the physical strength of the pellets and whether or not oil is leaking from the feed. Rather than focusing on the influence of the raw materials, this project dives into the very last centimetres of the extruder, *i.e.* the dies, and how they impact the PPQ: Will a better understanding of the pore structure allow for an improved physical pellet quality, and can the dies be utilized better to not only form the functional shape of an extrudate? That is the question to ask.

Even though a PhD study is executed independently, the work would not have been the same without the support of good friends, fellow students and collaborative partners. In that context, I will very much like to especially thank Bjarke T. Hansen from the Niels Bohr Institute for the alignment of cross-sectional images captured by µCT as well as Søren Mellemkjær Jensen for his laborious proofreading, valuable feedback and eminent home-made hot dogs. I have had plenty and ever-changing supervisors and co-supervisors throughout the project: Michael Engelbrecht, Stina Frosch, Bo Jørgensen, Flemming Jessen, Aberham Feyissa and Niels Hjermitslev – also known as Engelbrecht *et al*. Thank you all, and in particular, thank you, Aberham for your guidance and knowledge sharing, and you, Michael for your indefatigable supervision and our late brainstorming sessions at Berlin Bar.

Herlev, July 31, 2017
Markus Wied Dethlefsen

List of Publications

Paper I (enclosed in the public and confidential thesis)

Dethlefsen, M.W., N.H. Hjermitslev, S. Frosch & M.E. Nielsen (2016): Effect of storage on oxidative quality and stability of extruded astaxanthin-coated fish feed pellets. *Animal Feed Science and Technology*. Vol. 221, pp. 157-166.

Patent Application I (enclosed in the confidential thesis, only)

Title:	Method and device for producing a fish feed containing probiotic bacteria
Application No.:	EP16180323
Date of filing:	July 20, 2016
Inventors:	Markus Wied Dethlefsen
	Niels Harthøj Hjermitslev

Patent Application II (enclosed in the confidential thesis, only)

Title:	Nozzle for an extruder
Application No.:	EP17153567
Date of filing:	January 27, 2017
Inventors:	Markus Wied Dethlefsen
	Niels Harthøj Hjermitslev

Summary in English

The present thesis, *Die Hard – Improving the Physical Quality of Extruded Fish Feed Pellets*, approaches some of the biggest challenges within production of high-performance feed: Oil leakage and pellet strength. Salmon farmers in the aquaculture industry are requesting high energy dense diets with a supreme physical quality. To fulfil the market expectations, feed pellets have to contain 40% fat and tolerate high levels of stress during the transportation to the fish cages – without the pellets crumbling and oil leaking out of the feed. To solve this task, an improved understanding of the pellet structure's impact on the physical quality of the feed is required. Through detailed analyses of the pellets' microstructure, it was found that the optimal pore structure is defined by a high pore-surface-area to object-volume ratio. To obtain this pore structure, a new generation of dies was developed. These dies are proven to significantly reduce oil leakage while the overall pellet strength is significantly enhanced. The observations leading to the development of the new dies are published in the enclosed *Paper I*, whereas an application increasing the utilization of the pores is filed as *Patent Application I*, and the die technology facilitating an improved pore structure of extruded feed is filed as *Patent Application II*.

Sammendrag på dansk

Denne afhandling med titlen *Die Hard – Forbedring af den fysiske kvalitet af ekstruderede fiskefoderpiller* tager udgangspunkt i nogle af de største udfordringer inden for produktionen af højkvalitets foder, nemlig olielækage og pillestyrke. Lakseopdrættere i akvakulturindustrien efterspørger energitæt foder med en uovertruffen fysisk kvalitet. For at leve op til kundernes forventninger skal foderet indeholde 40% fedt og kunne modstå kraftige ydre påvirkninger på dets vej til fiskeburene – uden at pillerne smuldrer til krummer eller lækker olie. For at løse disse udfordringer er det nødvendigt at opnå en bedre forståelse af pillens strukturelle betydning for den fysiske kvalitet af foderet. Efter nøje undersøgelser af forskellige pillers mikrostruktur, har det kunnet konkluderes, at den optimale porestruktur er defineret ved et stort poreoverfladeareal set i forhold til det tilhørende pillevolumen. For at opnå denne struktur har det været nødvendigt at udvikle en ny generation af dyser. Disse dyser er bevisligt i stand til signifikant at reducere olielækagen, samt signifikant øge styrken af pillerne. De observationer, der har ledt til udviklingen af de nye dyser, er blevet publiceret i den vedlagte artikel. En metode til anvendelse af porerne er blevet indsendt som den første patentansøgning, mens selve dyseteknologien, der anvendes til dannelsen af den forbedrede porestruktur i ekstruderet foder, er blevet indsendt som den anden patentansøgning.

Abbreviations

API	Active Pharmaceutical Ingredient
CFD	Computational Fluid Dynamics
CFU	Colony Forming Unit
DORIS	Durability on a Realistic Test
DTU	Technical University of Denmark
ECS	Expansion Control System
FCR	Feed Conversion Rate
Je	Jesus Number
KIT	Karlsruher Institut für Technologie
PPQ	Physical Pellet Quality
R&D	Research & Development
Re	Reynolds Number
Re_c	Critical Reynolds Number
S:V	Pore-Surface-Area to Object-Volume
SF	In-house oil leakage test
SLS	Selective Laser Sintering
SPC	Single Pellet Coating
TA	Texture Analyzer
TVT	Texture analysis by Perten's TVT line
Ø	Diameter
µCT	Microtomography

1. Motives

All too often, you do stuff without asking "Why?". As you are not always in a position capable of having the mastery of issues, it is recommendable now and then to trust the validity and relevance of requests and proposals from more experienced fellow citizens – whether it happens to be your parents, supervisor or the local meteorologist. In your daily activities, the worst scenario would be to get rain-soaked from not bringing an umbrella. But when you start a three-year research project, you must carefully consider how to execute your tasks and communicate your findings. Never go along with the general practice if it does not feel right, and never apologize for doing things differently. However, when a given norm is fully adapted – *e.g.* structure of reports or minimum number of scientific references in the bibliography – everything diverging seems to need a level of justification. Or at least an explanation. However, the quality of the work should never be jeopardized by doing things differently, but the risk of failing when using traditional methods is striking if you thoughtlessly imitate your predecessor. Instead of dogging footsteps, make your own discoveries while respecting others, and combine it all with the attitude enunciated by Isaac Newton in his letter to Robert Hooke, February 5, 1675[1]: *"If I have seen further it is by standing on ye sholders*[2] *of Giants"* (Newton, 1675).

This chapter is nothing like an apology. It neither serves as a transcript of Newton's letters. It simply aims at explaining the *whys*. This by specifically drawing the attention to the topics: structure, scientific writing, references, realities, results, and focus.

1.1. Structure

Chaos is not necessarily the opposite of structure, and structure is no guarantee of meaning. Neither is chaos equivalent to rubbish. The present thesis is not intended to be chaotic; it just has an alternative structure. Traditionally, most theses within natural science and engineering consist of a detailed introduction to the conducted research, which is reported as enclosed papers and manuscripts. This layout is well-known, and thereby defined as structured. However, the present work is definitely more suited for a monograph, and will, consequently, involve alternative management of formalities and dissemination in general, cf. the *why*-paragraphs listed below. The aim of this approach is to create the framework for the reader, while being guided, to explore the field individually. In the end of this

Fig. 1: Advertisement from DTU exemplifying the phrase: *"Forstå det. Gør noget ved det."* (*"Understand it. Do something about it."*). (MetroXpress, 2013).

[1] Date as written in the original letter following the Julian calendar. Equivalent to February 15, 1676, using the Gregorian calendar.
[2] In the original letter written as *"ye sholders"* in the meaning of *"the shoulders"*.

thesis, the reader is supposed to phrase thoughts and ideas similar to the ones presented in the enclosed publications. This thesis structure is supposed to be very modern and educational, and is put into practice – and even used in advertisements, cf. *fig. 1* – on a daily basis at DTU, Technical University of Denmark, following the expression: *"Understand it. Do something about it."* Conforming to this mantra – and executing the commands in the listed order – induces scientific problem-solving and has been a keystone throughout the present project. In combination with the applied monographic structure, the reader will stay on the sideline while acquiring ever more topic-related insight (first part of the mantra), and finally be in a position capable of solving the challenges independently (last part of the mantra); identical to my own learning process, except the risk of getting as foul-smelly as a fish feed factory. One reason for primarily provide the reader with the theory needed to solve similar challenges independently is directly related to the confidentiality of the patent applications. Presenting the principles instead of detailed results make the thesis relevant to a broader audience, while no secrets are disclosed. In conclusion, the structure is also very much related to the task, and executing an industrial PhD is, if anything, more an applied discipline than comfying up full time at the university.

1.2. Scientific Writing

Each and every supervisor will recommend their students to follow an intensive course in scientific writing. I attended one myself. And no doubt, considering the abundance of flaky butter croissants and chocolate milk available throughout the lectures, the registration fee was peanuts and the teacher a volunteer activist. At this stage of the paragraph, I have already committed several scientific writing-crimes, like unformal phrasing and referring to myself as a human being. Of course, it is at all times important to retain a scientific level – and pay tribute to the communicative traditions, if they are contextual suitable – and the thesis will not transform into a diary, but there is no reason to refer to oneself using third person or the plural of majesty, when all of us know who is writing. Why insistently enforce the use of affected or anachronistic wording, if you risk losing half of your audience in the process of decrypting your message into plaintext? The goal is to mediate the findings to scientists as well as operators. Furthermore, having granted funding from Innovation Fund Denmark, I am obliged to meet their demands regarding Open Access Policy of June 21, 2012, by making the research available for everyone (Djurhuss *et al.*, 2012). Communication is the link between accessibility and availability, and hopefully, this link stays intact all along the forthcoming pages.

1.3. References

For centuries, *less is more* has been a popular statement[3]. However, this minimalistic approach should not be carelessly applied in any context. And in relation to publication of papers in scientific

[3] The phrase is written in the poem *"Andrea del Sarto"* by Robert Browning, 1855.

journals, quantity appears to be way ahead of quality regarding references in the bibliography[4]. As a result of the present thesis structure, it would not make sense to incessantly refer to external publications or enclosed manuscripts – partly because it would conflict with the idea of creating a framework for the reader to explore the field individually, partly because the majority of relevant references related to the published *Paper I* already are listed in the corresponding bibliography. Naturally, relevant literature will be used in the introduction as well, but not with the purpose of boosting the number of references. A lot of literature has been digested during the last years, but mainly as inspiration, amusement and to reach the contemporary scientific level – and in the end finally stand on the shoulders of my peers, to be Newtonian. Another equivalent important reason for reduced occurrence of references is the fact that two thirds of the publication list are patents with documented levels of novelty. Consequently, the references related to the patents are to a greater extent supporting mechanisms and models applied to specific setups rather than explaining similar or identical systems. In other cases, own observations form the basis of the chain of thoughts, and act to my knowledge as – though internal – the most reliable information source available.

1.4. Realities

Whether you consult courses in *Theory of Science* or the first movie in The Wachowski Brothers' *The Matrix* trilogy, the definition of reality is up for discussion. Only relying on Morpheus' definition of *real* as *"electrical signals interpreted by your brain"* (Wachowski & Wachowski, 1999) would hardly be fully adequate. Instead, and in the present project, the scientific reality is addressed as the – based on common knowledge – most qualified guess attainable given the information available at that specific time. This implies that the conceptual reality is not steady and can change when more data or better measurements are accessible. Until then, the intervening period serves as the highest level of realism and will, consequently, be defined as the current truth; a truth that changes continuously as more of the yet unexplored physical reality is revealed and explained.

1.5. Results

Big Data is the latest buzzword embraced by industries and universities, and it seems to be more resistant than the last dozen of buzzwords. But what to do, when big gets too big? Just the visualization of extrudate structures generated in this project more than one million files, spreadsheets with more numbers than available rows[5], and hard drives storing terabytes of data. Of course, this should, cf. *1.4. Realities*, vouch for a very reliable reality, but paying equally attention to each piece of information is unrealistic when working independently. Instead, prioritization of results and analysis of hot spots are better and more realistic approaches. In practice, the selection of data depends on its relevance and ability to induce clarification, render probable correlations, and

[4] Just my own experience. I accept the tradition, but allow myself to challenge it in some parts of my work.
[5] *I.e.* more than 1,048,576 rows in Microsoft Excel 2010.

draw conclusions. This is not the same as removing pseudo-outliers, as the exclusion takes place before the attempt to fit the data to a specific model, like removing cobblestones in a heap of apples with the purpose of describing the correlation between the volume and weight of apples. One could say that, given the analysis cost of one single extrudate, *i.e.* a fish feed pellet, adds up to 2,500 DKK, the budget for the expelled samples should ideally have been spent on representative pellets, as unused analyses reduce the potential size of the data set and, by that, the statistical significance.

Whether you are working at universities or in the industry, the encouragement to deliver results is very similar: receive grants or save/earn money, respectively. Even so, applications are most often supported by promises of saving the world by reducing global warming. When the economical and magnanimous motives are compatible, the industry, at least, will tell the investors about the savings while convincing the customers of its honourable and conscientious engagement in social responsibility issues. However, not being honest regarding the true motive can potentially affect the decision-making in the process of whether or not the findings should be implemented. An even bigger risk is – in the fight for success, though temporary – to be too narrow-minded and neither look one step back nor forward when searching for solutions. A quick fix at your desk can create even bigger problems later on in the production line if you are not paying attention to the impact on final product quality by changing an additive or adjusting a process parameter.

1.6. Focus

There are already plenty of handbooks and other literature explaining all steps in extrusion of feed, from raw materials to packaging. As both of these and, not least, all the unit operations in between, affect the final product quality, it would make sense to explain likewise. However, it would also be unnecessary, partly because most of the literature is available online or at libraries, partly because the reader is most likely acquainted with the principles of operations already. Instead, relevant operations will be described when required, and the primary focus will be on the very last part of the extruder, *i.e.* the dies, and how to physically utilize the entire potential of the extrudate. In the process of understanding the challenges ahead within development of fish feed, one have to know what to look for and focus on, cf. *fig. 1*. Over the next pages, the main challenges with respect to the physical quality of high performance fish feed will be presented and examined. The content will be based on the current development stage at BioMar A/S, but the challenges are present in all aquaculture industry dealing with energy dense diets. In an overall perspective, the focus of the thesis is characterized by the environment of the host company, BioMar A/S in Brande, Denmark, and the tasks that have been assigned to the group I have been affiliated to, the Process Innovation Team. As high energy dense diets are requested by all salmon farmers, one of our high-priority tasks have been to increase the oil inclusion while decreasing the oil leakage level. This focus will, consequently, imbue most of the thesis.

2. Fish Feed History and Future

The demand for fish feed arrived very shortly after the launching of fish farming, which dates back several thousand years to the Romans, Etruscans and Phoenicians (Brown, 1977). No details of volumes are available all back to the beginning, but The World Bank (2013) provides historical data from 1984 and projected levels of global fish production until 2030, see *fig. 2*. However, these data do not take the feed conversion rate (FCR) into account, but even significantly improved FCR's will not erase the indication of a growing industry, whether focusing on the feed or fillet.

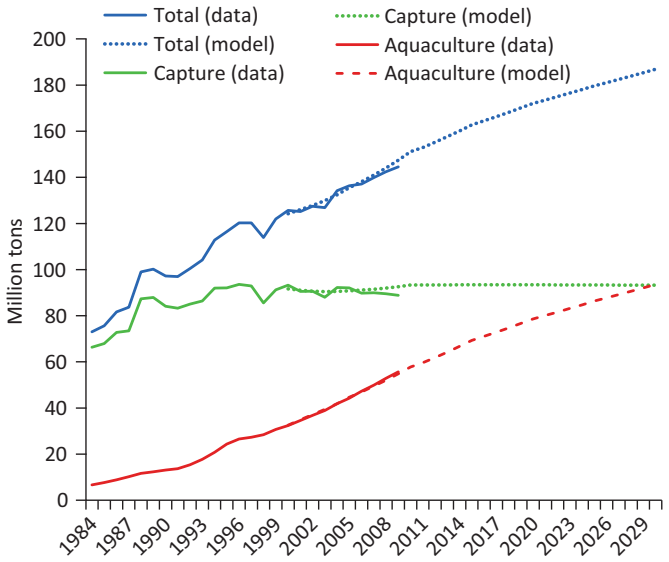

Fig. 2: Global fish production deriving from capture and aquaculture. Data and projections 1984-2030. (The World Bank, 2013).

Alltech (2017) estimates the global feed production in 2016 to have exceeded 10^{12} kg – divided among swine (26%), ruminants (22%), poultry (44%), aquaculture (4%), pet (2%), equine (1%) and others (1%) – and its total value to be around 460,000 million USD. With reference to aquaculture, 4% corresponds to $39.9 \cdot 10^{9}$ kg, but its value is estimated to 9% (Alltech, 2017). This is mainly the result of higher production costs of fish feed compared to most other feed types, which is partly caused by supply and demand of expensive raw materials, partly the result of harsh regulation requiring high performance feed having FCR's below 1.0 (which explains the need for high quality raw materials with high digestibility). Another reason for the more expensive ingredients for salmonid fish feed compared to feed for farm animals, is its higher content of proteins of animal origin, which is required as farmed fish are primarily carnivores, whereas farm animals primarily are herbivores or omnivores (Hjermitslev, pers.comm. 2017). Additionally, fish meal prices have risen and were almost doubled from 694 USD in 2005 to 1,379 USD in 2006 (Tacon & Metian, 2008).

The FCR limit originates from Dambrugsbekendtgørelsen[6] and was announced in 1989. On the one hand, this restriction was for the benefit of the environment as the increase in feed digestibility

[6] Means *"The statutory order regarding fish farming"*. Published as Announcement 224, April 5, 1989, Denmark.

reduced the discharge of nitrogen and phosphorous, while it on the other hand increased the farmers' feed costs (Tacon & Forster, 2003; Jokumsen & Svendsen, 2010). However, the farmers were assigned specific feed quotas and were – within this quota – allowed to produce as many fish as possible, on condition that they fulfilled the statutory order. This moved the focus from farming as cheap as possible towards farming as efficient as possible. At that time, Dansk Ørredfoder A/S (now BioMar A/S) was owned by the farmers, and this cocktail (FCR restriction, quota limit on feed but not on fish, and farmers as shareholders) made the national conversion of fish farming affordable. This new generation of high performance fish feed proved to be worth the extra cost, and soon, the rest of the aquaculture industry conformed to the Danish standard (Hjermitslev, pers.comm. 2017).

Ever since, farmers have inquired more and more energy dense diets – especially for salmonid feed – and now, the feed companies are struggling with recipes that were totally out of reach 20 years ago. Today, it is not uncommon to expect grower-feed to contain 35% protein and 40% fat; and even though it is possible to produce, the demands on the physical pellet quality (PPQ) are more pronounced than ever. Historically, fish feed has been manufactured in many different ways, going from fresh and chopped fish with very short shelf-life, to pelletized, expanded, or extruded feed with reduced water content and extended shelf-life (Brown, 1977). All of these production techniques are still valid, but extrusion is currently the only method to manufacture high performance feed with sufficient expansion allowing for 40% fat. For that reason, extrusion is the technology of interest in this project.

3. The Challenges

It is well-known that the ingredient blends for extruded fish feed are way more complex and heterogeneous compared to extruded polystyrene or aluminium constructions. The raw materials are varying from recipe to recipe, and even within the same production, it can be necessary to make adjustments when one ingredient runs out and a new lot must be used. Also, BioMar, specifically, has changed formulation philosophy to guarantee a defined feed performance rather than a fixed recipe, *e.g.* changing from one protein source to another without affecting the nutritional performance of the feed. This allows for flexibility if one ingredient either rises in price or is running low. Consequently, it is impossible to fine-tune the extruder to one single setup as recipes are ever-changing, and what you do today is not necessarily applicable tomorrow. In combination with high customer expectations regarding fat levels and durability of the feed, the main challenges, which all are related to the PPQ, are very much identical to the top five in BioMar's statistic listing customer claims. The data originate from an internal report from 2014 only covering the Norwegian production and are sorted in descending order accordingly to the size of the compensation and not the number of claims. The amount of affected feed and the corresponding compensation divided among the top five are listed in *table 1*.

Table 1, The five most costly quality issues related to the PPQ. Data only cover the Norwegian production in 2014, and are sorted in descending order according to the size of the compensation.

Top Five	Affected feed $[\times 10^3 kg]$	Compensation $[\times 10^3 NOK]$
1. Oil leakage	3,815	2,483
2. Floating	2,669	1,089
3. Dust	545.1	238.2
4. Size	137.7	117.1
5. Strength	255.3	97.47
Total	**7,422**	**4,025**

Originally, most of the challenges related to the PPQ were intended to be handled by the Process Technology Group (now Process Innovation Team) in internal BioMar R&D projects. Within the military, terrorist organisations and intelligence agencies, operations and projects are most commonly assigned with code names, and BioMar A/S is no exception. Three major projects, *Black Box*, *Exactus*, and *Opticon* formed the basis for the present project that originally was entitled *Physics of Extrusion* but later on turned into *SmartPore*. It is no shame to be ambitious, and *Black Box*, *Exactus*, and *Opticon* were indeed the result of high ambitions. The aim was to model and explain the entire extruder, which was considered as a "black box", and develop software to predict everything going on from inlet to outlet. This turned out to be too much to swallow, and slowly but surely, the projects were transformed into a lighter edition: *SmartPore*. Instead of focusing on the entire extruder, we started from the very end, *i.e.* the dies, and decided not to go much further back, as these last centimetres turned out to have a major impact on the PPQ. In the following chapter, the *top five* along with related parameters relevant to the PPQ will be examined.

4. Physical Pellet Quality Parameters

To understand the forthcoming PPQ parameters of interest, some background information covering extrudate expansion, nucleation, and pellet formation is needed. These topics have already been treated in details in my master's thesis from 2013, and its dissemination is still equally valid. As the associated concepts play an important part in the subsequent paragraphs, and to make the reading comfortable, the relevant passages from my previous work have been slightly modified and then pasted into this chapter. This applies to the paragraphs: *4.1. Expansion*, *4.2. Nucleation* and *4.3. Pellet Formation*, and to some extent to *4.5. Pellet Shape*.

4.1. Expansion

Even though the shape of the die and revolution speed of the cutter create the framework for the size of the extrudate, the expansion highly influences the properties and final appearance of the pellet (Moraru & Kokini, 2003). As the growth rate of fish from the aquaculture industry is related to the size of their feed (Wankowski & Thorpe, 1979; Azaza *et al.*, 2010), it is important to understand the expansion mechanisms.

The driving force in pellet expansion is the vaporization of moisture (Moraru & Kokini, 2003; Cheng & Friis, 2010). During extrusion, an overpressure is build up inside the barrel. Consequently, even though the temperature exceeds 100 °C, there is no vapour present. First when the extrudate leaves the die, the moisture vaporizes and expands the pellet during pore formation (Williams, 2000). Therefore, it is reasonable to deduce that a high content of moisture boosts the expansion potential. However, increasing the moisture content will not decrease the bulk density as the pellet collapses after the initial expansion (Ding *et al.*, 2006). This is due to the concept of puff-drying introduced by Rossen & Miller (1973)[7]: During the expansion – *the puff* – caused by the vaporized moisture, the pellet ideally gets rigid immediately as it *dries*. If the moisture content is too high, the drying is delayed and the pellet contracts before it hardens (Williams, 2000). The contraction is shown in *fig. 3*, and will be further described under *4.2. Nucleation*. To minimize the vaporization from the extrudate, a vent can be adjusted to flash-off moisture before the melt exits the die (Riaz, 2000).

Many different and interacting parameters such as die temperature, screw speed and added moisture are involved in the expansion process (Cheng & Friis, 2010). To understand the mechanisms, it can be overwhelming to include all parameters at once. Therefore, it is practical to only consider the viscoelasticity of the melt – at least for a start. This entity, combining viscosity and elasticity in one word, summarizes many of the underlying parameters. In contrast, the expansion is often split up in two: The axial[8] and radial[9] expansion. Assessing the two directions of expansion

[7] Rossen & Miller (1973) defined the present type of extrusion by: "Food extrusion is a process in which a food material is forced to flow, under one or more of a variety of conditions of mixing, heating, and shear, through a die which is designed to form and/or puff-dry the extrudate."

[8] Alvarez-Martinez *et al.* (1988) call this for the longitudinal expansion.

separately, Launay & Lisch (1983) concluded that the axial and radial expansion depended on the viscosity and elasticity, respectively: Decreasing viscosity increases the axial expansion, and decreasing elasticity decreases the radial expansion. They also concluded that the two directions of expansion are negatively correlated; thus, the viscosity and elasticity are positively correlated.

The final pellet is the result of a struggle between inwards and outwards forces. Even though these forces are conflicting, there is no conqueror; the degree of expansion is essentially a negotiation. *I.e.*, as the melt leaves the die, and the moisture vaporizes, the bubbles from the nucleation start to expand and will constitute the primary outward force. Simultaneously, inward forces – due to the viscoelasticity and flash-off or rupture of the bubbles – restrict or limit the expansion (Kokini *et al.*, 1992; Moraru & Kokini, 2003).[10]

The development of the extrudate can be listed stepwise and is often illustrated as in *fig. 3*. The five steps are based on Kokini *et al.* (1992) and Robin *et al.* (2012), and enhance the understanding of the expansion. The first step is not shown in *fig. 3* and takes place prior to the die, whereas the remaining four steps will be unveiled under *4.2. Nucleation*. At this stage, the listing and figure serve as a summary of presented and forthcoming theory, and emphasize the order of and terminology in extrudate development:

1. Transformation of the feedstock into a uniform viscoelastic melt. *[not shown in fig. 3]*
2. Nucleation of bubbles.
3. Initial swelling of the extrudate due to the elasticity of the melt and release of pressure.
4. Growth of bubbles and expansion.
5. Contraction when the wall of some bubbles no longer can sustain the internal water vapour pressure.

To understand *fig. 3*, it is important to know that the extrudate represents a fraction of an infinite solid, and that all cross-sections undergo the identified steps. The shape of the extrudate changes when the cutter divides the infinite solid into pellets, cf. *fig. 5*.

[9] As well referred to as the sectional expansion (Cheng & Friis, 2010), cross sectional expansion (Alvarez-Martinez *et al.*, 1988) or the diametral expansion (Kokini *et al.*, 1992).
[10] This example is simplified, and further details will be presented under *4.2. Nucleation*.

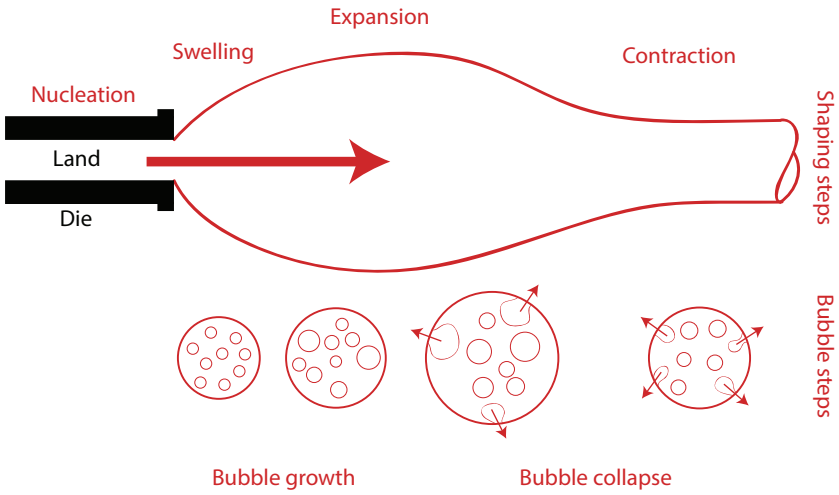

Fig. 3
The prevalent way to illustrate development of the extrudate from nucleation to contraction. Black is used for terms and parts related to the extruder, while red is used for processes in relation to the extrudate. The radial cross sections (bottom) are downscaled proportional to the axial cross section (top). Based on Kokini *et al.* (1992), Moraru & Kokini (2003), Guy (2001) and Williams (2000).

4.2. Nucleation

If the vaporization of moisture is the driving force in pellet expansion, then the nucleation is the necessary catalyst, which exposes the underlying mechanisms. Despite the importance of nucleation, being responsible for the structure of the extrudate, only few studies are available. To explain this schism, Moraru & Kokini (2003) emphasize the complexity of the phenomena as the obvious reason for the lack of research. Therefore, the theory is very often based on simplifications and assumptions.

Basically, nucleation is the formation of small, thermodynamically unstable and gaseous bubbles in the melt. The driving nucleation emerges from air bubbles retained from the starch granules and entrapped during extrusion (Moraru & Kokini, 2003). These bubbles, called hilums and with a diameter of about 0.5 µm (Moraru & Kokini, 2003), are expected to decrease in number if the granules are highly dispersed in the extrusion process (Guy, 2001). As shown in *fig. 3*, the nucleation takes place in the land of the die. Frame (1994) presents different and more exotic modifications of the land – but for now, the design is kept simple. Due to the heterogeneous composition of the feedstock, the nucleation, and therefore the bubble growth too, is not uniformly distributed in the melt and, consequently, the structure of the extrudate will be heterogeneous as well (Liu, 2005; Moraru & Kokini, 2003).

As indicated, the vaporization is the driving force in expansion. Considering that the phase shift essentially is caused by the pressure drop after the die exit, Kokini *et al.* (1992), however, specify that the difference between the vapour pressure at the extrusion temperature and the atmospheric pressure constitutes the driving force. As a result of the increasing temperature from the first to the

last section of the barrel[11], this pressure difference appears before the melt reaches the die. Also, the hilums are present in the feedstock from the beginning. Therefore, it is obvious to ask, why the nucleation primarily occurs in the land. The answer is very closely related to the rheological fact that the melt is shear-thinning; in relation to fish feed, Lam & Flores (2003) report on a power law exponent, n, of 0.246. Consequently, the melt is non-Newtonian and has an apparent viscosity, η, which decreases with increasing shear rate, $\dot{\gamma}$ (Moraru & Kokini, 2003; Risum & Friis, 2009). Also, as it emerges from *fig. 4* and *equation 1*, η decreases with increasing shear stress, τ. m is the consistency index and has the unit $\mathrm{Pa{\cdot}s}^n$.

$$\eta = m \cdot \dot{\gamma}^{\,n-1} = m^{\frac{1}{n}} \cdot \tau^{\frac{n-1}{n}} \qquad (1) \qquad \textit{[based on Risum \& Friis (2009)]}$$

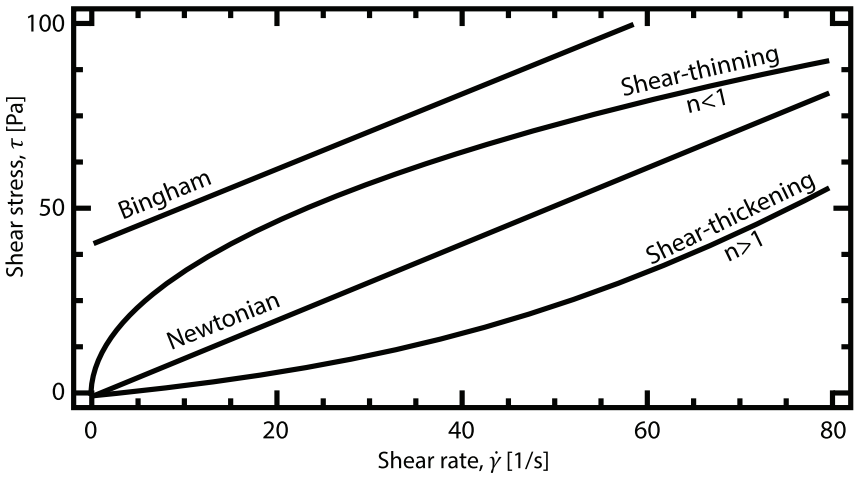

Fig. 4
Flow curves for liquids with different behaviours. The apparent viscosity, η, is given by the slope. Shear-thinning and shear-thickening are also referred to as pseudo plastic and dilatant, respectively. Based on Risum & Friis (2009).

The shear rate and shear stress originate from the rotational speed of the screw in the barrel, and the pressure within the screw, respectively (Kazemzadeh, 2011). The land in the die constitutes a clear reduction of the cross-sectional flow diameter. Therefore, when the melt enters the land, τ increases and η decreases. The reduction of the apparent viscosity favours the ongoing nucleation and results in the growth of bubbles (Ding *et al.*, 2005). Regarding the viscoelastic properties and due to the shear of the melt, the viscous character is predominant inside the barrel and in the die. Therefore, the elastic impact is often neglected for the flow in the land (Moraru & Kokini, 2003). However, the elasticity is closely related to the swelling after the die exit and will be further developed below.

Simultaneously with the growth of bubbles in the land, the melt is moving towards the die exit. This combination of shear and nucleation generates elongated bubbles parallel to the flow, and results in axial expansion (Ma *et al.*, 2012). This is in accordance with Moraru & Kokini (2003) and Launay &

[11] This was the case for the pellets produced during the master's thesis.

Lisch (1983), who, respectively, established that the viscosity is predominant in the region of nucleation, and that decreasing viscosity will increase the axial expansion.

Because the apparent viscosity decreases with increased shear, the nucleation and expansion are more pronounced for high *L*/*D*-ratios of the die (Moraru & Kokini, 2003). However, the expansion decreases if the land is too long (Williams, 2000). This is due to fouling of the land and burning of the product (Frame, 1994), giving a denser and less expandable extrudate. In general, when the diameter of the die increases, the radial expansion decreases as a result of decreased shear and swelling (Sokhey *et al.*, 1997). When small extrudates are wanted, this can give problems since blocking of the channels are more likely to happen using tiny cross-sectional dies (Guy, 2001). Hereby, the radial expansion increases along with the nucleation and swelling in the unblocked die channels.

Based on *4.1. Expansion* and the currently presented nucleation theory, it can be summarized that the viscosity is succeeded by the elasticity when the melt emerges from the die. As shown in *fig. 3*, this is also the time of swelling, which is why elasticity and swelling often are treated collectively (Moraru & Kokini, 2003). The swelling is the result of accumulated or suppressed elastic forces in the melt after being pumped through a small die channel (Guy, 2001; Moraru & Kokini, 2003), and will affect the radial expansion directly. This also explains the correlation between elasticity and radial expansion presented by Launay & Lisch (1983). However, only few references are available regarding extrusion and swelling. Miller (1963) argues that the reason for the lack of literature may be due to none obvious production issues or because the issues are not understood.

The radial expansion is driven by the elasticity and bubble growth, and stabilized or restricted by the shift in viscosity and collapse of expanded bubbles. The formation of bubbles is the result of nucleation, and the growth of them is encouraged by the elasticity and temporary decreased viscosity (Launay & Lisch, 1983; Ding *et al.*, 2005). As the shear decreases after the die exit, the apparent viscosity increases and contributes to the settlement of the extrudate (Miller, 1994). When the bubbles reach their limit of extensibility, they rupture, and the extrudate starts to contract under elastic recoil (Guy, 2001). During rupture, the vaporized moisture is released and favours the drying of the extrudate (Guy, 2001; Moraru & Kokini, 2003). As emphasized under *4.1. Expansion*, increased moisture content will probably increase the initial expansion, but also increase the contraction due to the delayed drying.

4.3. Pellet Formation

As shown, this introduction to extrusion theory limited to the die is extensive and implies a lot of different mechanisms. *Fig. 5* is compiled to summarize the moment of pellet formation by combining the presented theory and illustrations. Even though the processing within the barrel highly

influences the extrudate, *fig. 5* is mainly based on *fig. 3* as expansion and nucleation are alpha and omega regarding pellet formation.

Without restrictions, the pellet would inflate into spheres during extrusion (Miller, 1994). However, during swelling and initial radial expansion, the pellet top (first point exiting the die) assumes a spherical shape (convex), whereas the bottom (last point exiting the die) turns flat or even concave as the result of knife flattening and decreasing expansion potential after pore rupture as shown in *fig. 5*.

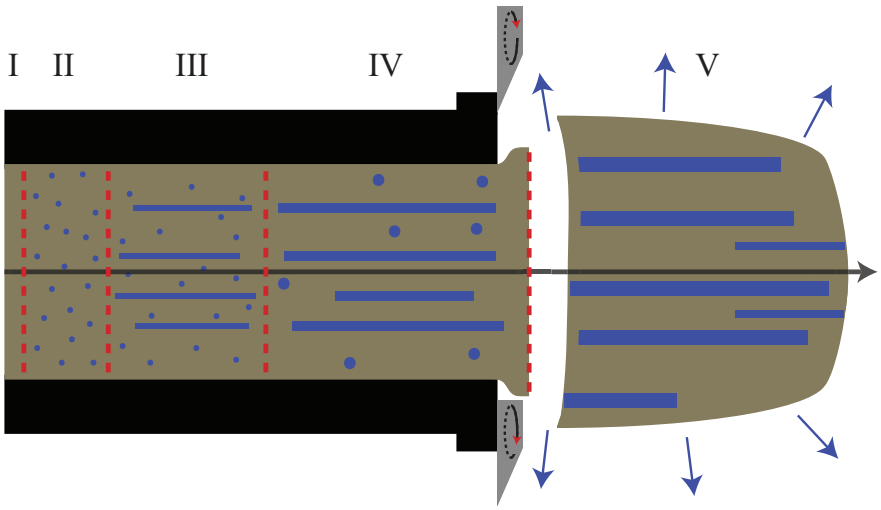

Fig. 5
Transformation of melt into pellets. Blue is used for nucleation, pores and water vapour flash-off. Red is used to show where the (grey) knifes cut the extrudate into pellets as soon as it exits the die.

I: Melt entering the land. II: Initial nucleation. III: Further nucleation, shear, elongation of bubbles, and axial expansion. IV: Further axial expansion; swelling and initial radial expansion appear as the extrudate exits the die. V: Cut off and pellet formation.

The left and right part of the final pellet is denoted bottom and top, respectively. The top starts to expand spherically when the moisture vaporizes. When the bottom is cut, the pellet expansion potential is accordingly reduced as the majority of the vapour has already left the pellet throughout its top and site; thus, the bottom appears to have an even or slightly concave shape.

4.4. Strength & Durability

From the moment the extrudate leaves the extruder, it has to withstand a lot of stress. In the beginning, the structure is soft, but as it dries and turns solid, the structural flexibility of the feed pellets drops. Hereby, the feed is more vulnerable to the harsh conditions constantly challenging its mechanical strength on its way to the fish: From stacking of bigbags in the storehouse, cf. *fig. 6*, and delivering to the customers in bumping trucks, to silos full of feed being transported by pneumatic pumps to the cages.

Fig. 6
Feed in stacked bigbags highly challenges the physical strength of the pellet structure. Karmøy, November 11, 2016.

If the pellet structure is too weak, the feed arrives as fines and dust, resulting in reduced growth, oil leakage, and customer claims, cf. *table 1*. Among many other papers, Sørensen *et al.* (2010) and Thomas *et al.* (1998) demonstrate and argue for the effect of ingredients on the physical strength of animal feed. Even though different sorts of starches and binders can increase the physical strength of the extrudate, you most often retain the same overall structure. Maybe, the average pore size has changed, but the direction of the pores is unaffected and will continue to be parallel to the flow direction in the die, cf. *fig. 5*. All scouts and most brave boys know about splitting wood with a dagger: splitting is easy – even with a blunt knife – as long as you do it parallel to the grains. And the same applies to fish feed pellets: They will most likely crack along the pores. As proved by Sørensen *et al.* (2010), the physical strength can be significantly improved by choosing the right starch source, however, imagine the effect by obtaining a pore structure more similar to gas concrete rather than the existing well-arranged parallel pores. Intuitively, isotropic pores should provide stronger extrudates than parallel pores, and following the gas concrete analogy, evenly distributed point-shaped pores provide, likewise, stronger extrudates than elongated pores.

4.5. Pellet Shape

The cutter transforms the infinite extrudate into pellets, and the arrangement and design of cutting knifes can shape the pellets into *"animals such as horses and dinosaurs"* (Guy, 2001). Cf. *4.1. Expansion*, the size of the pellet is important for the growth of the fish, but no literature can be found that proves the shape has similar effects. Therefore, the shape and likeness to animals are of primarily commercial interest. Likewise, Chessari & Sellahewa (2001) refer to the bulk density as an attribute important in commercial production, because the packages are filled by weight and not by volume: If the bulk density is to low, the packages will overflow. These considerations show that shape and density depend on the industry and the consumer: On a breakfast table, a sufficiently filled pack of funny, *Brachiosaurus*-shaped extrudates will create a relaxed atmosphere, whereas the

fish farmer associates the density with a parameter determining whether the pellet will float or sink; a parameter important for the availability of the feed (Riaz, 2000; Bandyopadhyay & Rout, 2001; Kraugerud *et al.*, 2011).

The pellet shape is not affecting the physical pellet quality directly; however, it highly influences the risk of dust and unintended floating. These issues are not necessarily the result of problematic geometries, and can as well be caused by intensive handling and entrapped air/too low density, respectively. Avoiding sharp edges, which primarily appear at the concave pellet bottom illustrated in *fig. 5*, will, nevertheless, dramatically reduce the associated problems (Hjermitslev, pers.comm. 2017). The dust is easily created from concave pellets as they rub against each other, and even when the pellets are dust-free when they leave the factory, there can be unacceptable levels of dust, when the feed arrives at the customer. The shape-related unintended floating is caused by surface tension and is mainly a problem for pellets in the range of Ø2-3 mm. From the ongoing internal quality control, it has been reported that pellets suffering from this type of floating most often are dangling from the water surface with the concave bottom pointing upwards and the convex top pointing downwards, whereby the pellet bottom acts like a boat and retard the sinking even further. Regarding the surface tension, the pellet utilizes the same floating trick as the *Gerridae*[12] walking on water. To brush-up this physical phenomenon, a particle will float awash – even with a density exceeding the liquid's – as long as its Jesus Number, *Je*, is above 1 (Hansen, 2003). *Je* is, cf. *equation 2*, calculated as the upward force, F_{up}, divided by the downward force, F_{down}. To calculate F_{up}, the circumference, *C*, of the particle in direct contact with the surface of the liquid is multiplied by the surface tension, *T*, of said liquid, whereas F_{down} is calculated as expected: the mass, *m*, of the particle multiplied by the standard gravity, *g*.

$$Je = \frac{F_{\text{up}}}{F_{\text{down}}} = \frac{C \cdot T}{m \cdot g} \qquad (2)$$

Fig. 7 has been computed to illustrate the relevance of the Jesus Number. Of course, the exact curve pattern will depend on a variety of factors, *e.g.* the specific feed type, *L*/*D* ratio of the pellet, and water characteristics such as temperature and salinity, but the tendency is unaffected: primarily small pellets will suffer from unintended floating.

[12] Family of insects, *e.g.* covering water bug and water skippers.

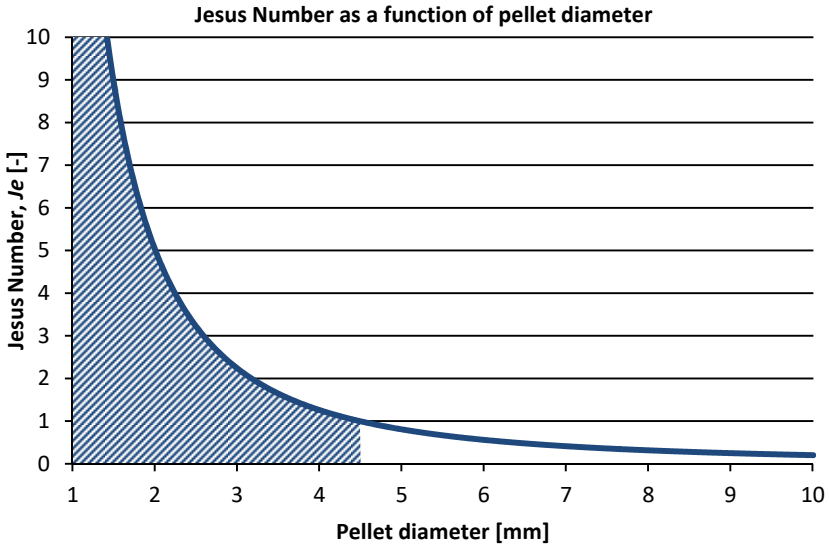

Fig. 7
Computed example plot of Jesus Number, *Je*, as a function of pellet diameter for typical cylindrical fish feed. Pellets will float when *Je* exceeds 1 (hatched area). This explains why primarily small pellets suffer from unintended floating.

In addition to the already covered axial and radial expansion, the flow characteristics of the dough throughout the die also have a major impact on the final extrudate appearance. Whereas the length and diameter, respectively, are mainly influenced by the two above-mentioned types of expansion, the flow behaviour is responsible for the shape of the pellet top and bottom, and determines their degree of convexity and concavity, respectively. Realising that the shapes of the extrudate extremities mimic the flow profile of the dough in the die implies that the flow has to be laminar; otherwise – in the case of turbulence – the pellet would tend to be way more cylindrical and without any convexity and concavity at the top and bottom, respectively. In *5. Test Procedures*, this statement will be further supported by rheological properties of the dough. Until then, we have to rely on the power law exponent reported by Lam & Flores (2003), cf. *4.2. Nucleation*, and the proclamation that the shape of the pellet top and bottom is caused by a laminar flow throughout the die. According to this paragraph, unintended floating and dust related to the pellet shape can be prevented if the Reynolds number, *Re*, is increased; however, increasing *Re* implies unrealistic changes in viscosity and linear flow velocity, cf. *5.6. Viscosity*.

4.6. Dust

Dust and fines attract a lot of attention in the aquaculture industry as they account for a wide range of problems, *e.g.* reduced feed nutrition, increased environmental pollution, extra maintenance of feeding systems, and additional costs among other customer claims. As described in *4.3. Pellet Formation*, *4.4. Strength & Durability*, and *4.5. Pellet Shape*, the dust is created during handling of the feed and is primarily caused by imperfect pellet geometries and fragile pellet structures. Even if the amount of dust is within the 1% limit and the average feed price is estimated to only 1 USD/kg (Jokumsen & Svendsen, 2010; Haubjerg, 2016), the loss for all aquaculture feed could amount to as

much as 400 million USD/year, cf. *2. Fish Feed History and Future*. The dust is lost to the sea and increases the discharge of nitrogen and phosphorous, and depletion of oxygen (Cornel & Whoriskey, 1993). Also, it highly challenges the pneumatic feeding systems, which also are co-responsible for the creation of dust by attrition and abrasion of the feed pellets when they are transported to the cages throughout hoses and pipe systems (Aas *et al.*, 2011). If the feed is also suffering from oil leakage, the combination of free oil and dust will inevitably result in blocked pipes, time-consuming cleaning, and claims. Therefore, the pellet has to be dust resistant and capable of resisting even harsh handling.

4.7. Floating & Sinking

Fish feed is, depending on the fish species, intended to either sink or float (Hjermitslev, pers.comm. 2017). Apart from the unintended floating related to the Jesus Number, cf. *4.5. Pellet Shape*, the feed's dynamic in water can easily be controlled by the bulk density, which often is adjusted by means of a degasser mounted on the extruder or an ECS (Expansion Control System) in direct continuation of the knife house. However, more and more customers are requiring semi-floating feed, *i.e.* 10-20% of the feed is floating while the rest is sinking. The primary reason for using this type of feed is to reduce waste related to overfeeding: At first, the fish species taking advantage of this type of feed start to eat the sinking pellets, and continue to the floating ones when no more feed is sinking down through the water. The dinner is over, when the floating feed is gone as well.

For semi-floating feed, the extruder is ideally adjusted to produce pellets with a specific density distribution; alternatively, batches of feed with different densities are blended afterwards. Previously, and still to some extent, Bredol® was applied to the pellet surface of entire batches to prevent floating by changing the surface tension of water. Theoretically, the degree of floating – especially for small pellet sizes, cf. *4.5. Pellet Shape* and *fig. 7* – could be very accurately controlled by blending one Bredol®-coated fraction of a batch with another Bredol®-free fraction of the same batch. However, the industry has, to my knowledge, never used this blending method – and controlling the degree of floating without additives is always preferable.

For sinking feed, specifically, there is one more concern: The sinking speed. If the feed sinks too slowly, the pellets can be wasted as they wash away, and if the feed sinks too fast, the fish have no chance to catch the pellets before they reach the bottom of the cage. The easiest way to illustrate the terminal sinking velocity, v, of a pellet having the density ρ_{p} and radius r, when sinking down through a fluid with the density ρ_{f} and viscosity μ, is by means of Stokes' law (Hansen, 2003), cf. *equation 3*. The standard gravity is denoted g:

$$v = \frac{2 \cdot (\rho_{\mathrm{p}} - \rho_{\mathrm{f}}) \cdot g \cdot r^2}{9 \cdot \mu} \qquad (3)$$

From an industrial point of view, only ρ_p and r are possible to adjust, but in reality, the only option is to change the density as the pellet size follows the size of the fish (Wankowski & Thorpe, 1979; Azaza *et al.*, 2010) – and varying the size rather than adjusting the density will, cf. *table 1*, lead to customer claims.

4.8. Oil Leakage

Oil leakage is by far the biggest PPQ issue within the fish feed industry, cf. *table 1*. Even if the feed looks fine in the warehouse, the bigbags can arrive to the customer soaked in oil. In addition to the inconvenience related to extra cleaning of trucks and farming sites, the oil can, cf. *4.6. Dust*, create major problems in the pneumatic feeding systems. To prevent degradation of heat-labile ingredients in the production of the feed pellets, heat-sensitive additives, *e.g.* astaxanthin, specific vitamins and amino acids, enzymes, and probiotics, are mixed with the oil and added to the pellets in the coating step. However, this means that not only oil is lost during oil leakage; the nutritional, functional and health-related feed properties are reduced as well. Most of these additives introduced in the coating are expensive, and the oil leakage is consequently a direct loss for the farmer. The depletion of astaxanthin from the feed can additionally cause insufficient flesh pigmentation at slaughtering, which negatively affects the value of the fish (Torrissen, 1985). Consequently, oil leakage, and ways to prevent or reduce it, has a lot of attention. Fat sealers, blocking the oil-filled pores, and emulsifiers, increasing the viscosity of the oil, are very common methods to reduce oil leakage, but even these chemically inspired technical aids can not stand alone when tomorrow's industry is demanding more than 40% oil in the feed. To solve this challenge, one solution could be to combine the chemical approach with innovative physical pellet properties fulfilling tomorrow's expectations.

4.9. Oxidation

The growth and health of fish are adversely affected if they eat oxidized feed (Hernández *et al.*, 2014). Some ingredients can be oxidative stable for years, but mixed together and passing through the extruder fire up the lipid oxidation and significantly reduce the shelf life of the final product. Especially the use of haemoglobin meal accelerates the oxidation as the iron initializes the Fenton reaction and thereby generates free hydroxyl radicals (Belitz *et al.*, 2004). Even though this problem is more related to the chemical pellet quality, it is necessary to look beyond chemistry to solve or minimize the current oxidation problem. For years, ethoxyquin has been the preferred antioxidant in fish feed; however, the lack of data on safety and exposure for target animals, consumers and the environment has made it impossible to assess the risk of using ethoxyquin. Consequently, The European Commission declared June 7, 2017 that ethoxyquin soon will be suspended and that *"feed materials and compound feed produced with the additive ethoxyquin or with premixtures containing it may continue to be placed on the market until 28 December 2017"* (EU, 2017). At the moment, no replacement as effective as ethoxyquin has been found. Instead, alternative methods to maintain the current shelf-life and minimize oxidation are in request – and "physical" thinking is very

reasonable to look into. Is it possible to pack the feed in an oxygen-free atmosphere using an oxygen-barrier-based laminate? Or can the feed itself act as a protective packaging material to minimize oxidation? Especially the last idea would be very elegant – to use the individual extrudate structures to not only retain the oil and heat-labile additives, but shield it from oxidation as well. Have this idea in mind when the next chapters reveal themselves.

5. Test Procedures

There exist numerous technologies and procedures to determine the physical pellet quality. There even exist many different test methods for the evaluation of the same physical quality. The lack of standardization is a big problem, as well as the fact that many of the methods are developed for terrestrial animals and not suitable for fish feed (Sørensen, 2012). As the aim is not to compare different test methods, only one per quality (except oil leakage) – reflecting the analyses conducted to evaluate and compare the pellets produced in the present project – will be presented in *this* chapter, and each of the presented methods is developed to evaluate the respective parameters presented in the *preceding* chapter (except oxidation). As the viscosity is needed as an input parameter in the next chapter, the associated results are presented directly in the present paragraph. The results obtained from other methods in this chapter will be presented when relevant. Regarding statistics, if nothing else is mentioned, average values are reported with one standard deviation, and samples are evaluated by an ordinary one-way ANOVA using the Tukey test in Prism 7.03 (GraphPad, California, USA). When only two groups are compared, an unpaired one-tailed t test is used instead. Groups are assessed as significantly different if the *P*-value is below 0.05.

5.1. Strength

Previously, a spring device commonly known as Kahl (Amandus Kahl GmbH & Co., Reinbek / Hamburg, Germany), see *fig. 8*, was the only instrument used in BioMar to determine the pellet strength. The Kahl test is time consuming and the quality, tightening speed and condition of the spring, determining the force needed to crack the pellet, highly influence the result. Consequently, the BioMar factories are changing to texture analysers, either from Perten's TVT line (Perten Instruments AB, Hägersten, Sweden) or from Stable Micro Systems Ltd, Godalming, United Kingdom. For the present analyses, the TA-XT plus Texture Analyzer (TA) from Stable Micro Systems mounted with a cylindrical probe (P/40) was used. The data were processed by Exponent Software from Stable Micro Systems, version 4.0.13.0, the probe speed was set to 0.10 mm/s, and the trigger force was 0.04903 N. The compression (distance) and force needed to crack the pellets were measured and used to compare the strength of different batches of pellets. In total, 100 pellets per batch were tested: 50 pellets exposed to the probe along the direction of the radial expansion (referred to as "curved") and 50 pellets along the direction of the axial expansion (referred to as "top-down"). The current internal quality criterion is 10 pellets exposed along the direction of the axial expansion (one by one) and in average withstanding 50 N.

Fig. 8
Kahl instrument still in function at BioMar SA, Pargua, Chile. For analysis, 10 pellets are measured. The result is given in kg.

5.2. Durability

The DORIS tester (Durability on a Realistic Test) (Akvasmart, AKVA group ASA, Bryne, Norway) is designed to mimic the pellet degradation during pneumatic feeding (Aas *et al.*, 2011). Even though there exists more realistic test methods, *e.g.* an entire simulation rig including a 20.4 kW blower generating up to 10.97 m^3/min airflow, air cooler, feed dosing system, and 400 m pipeline (Aas *et al.*, 2011), the DORIS is widely used in the aquaculture industry and takes up less space than a big test rig. A standard DORIS tester and the test procedure used within BioMar are shown and described in *fig. 9*. The result is between 0% and 100%, and corresponds to the mass fraction (*m*/*m*) of whole pellets after DORIS exposure relative to the initial sample mass. If nothing else is stated, the in-house laboratory runs the test in single.

Fig. 9
DORIS tester. A sample of 350 g of pellets is introduced in the left hopper, and then transported to the vane by an Archimedes' screw to be exposed to stresses corresponding to pneumatic conveying. Finally, the pellets are collected in a cup and the fraction of whole pellets is measured. (Aas *et al.*, 2011).

5.3. Oil Leakage

The in-house oil leakage test in BioMar (often referred to as SF, Norwegian abbreviation for centrifuge) is very standardized across the organization. Even the centrifuge model is the same; Allegra X-30 (Beckman Coulter, Indianapolis, USA). To evaluate the level of oil leakage, two drainable cups are each filled with 150 g of pellets and centrifuged at 123 *g* for 5 minutes. The result is measured in percentage, and corresponds to the mass fraction (*m*/*m*) of leaked oil relative to the initial sample mass before centrifugation. If nothing else is stated, the in-house laboratory runs the test in single. Most often, oil leakage levels up to 1% are accepted but even 5-10% is accepted if lower values for a period are not obtainable. However, the feed is then sold at a discount.

In addition to the SF test, I developed a Single Pellet Coating test (SPC) to obtain detailed information for specific samples. As a first step, the pellets to be tested are individually weighed (Mettler-Toledo AG204DR), then vacuum coated for 1 minute in a 500 ml vacuum flask connected to a vacuum pump (Vacuubrand MZ 2C, 1.7 m^3/h, Wertheim, Germany) while being fully submerged in 25 °C rape seed oil. Hereafter, the pump is turned off and the vacuum is instantly released. After the vacuum release, the vacuum cycle is repeated to ensure maximum coating. The time for each cycle is measured from the vacuum pump is turned on, and end when the pump is turned off. Next, the pellets are removed from the vacuum flask and wiped in kitchen roll until the surface is not glistening. Subsequently, the pellets are weighed once again and placed in an Eppendorf tube (1.5 ml safe-lock Eppendorf tube) with the pellet top pointing upwards as shown in *fig. 10*. Next, the tubes are centrifuged for 5 minutes at 50 *g* (Hettich Universal Zentrifugen 320R, Tuttlingen, Germany). After centrifugation, the bottom of the Eppendorf tube will contain the free oil. Finally, the pellets are weighed one more time. The result is measured in percentage, and corresponds to the mass fraction (*m*/*m*) of leaked oil relative to the mass of oil added during coating. If nothing else is stated, eight pellets per code are tested by SPC.

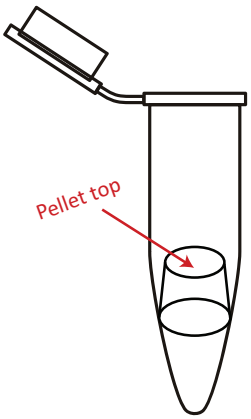

Fig. 10
Arrangement of pellet in an Eppendorf tube. Based on Clker.com (2017).

5.4. X-ray Microtomography / µCT

The microstructure of the pellets can be visualized using a wide range of different techniques. From high-definition digital imaging, to light microscopy, cryo-scanning electron microscopy, and scanning electron microscopy (SEM), which all are described in further details by Draganovic *et al.* (2013). The disadvantage by using these techniques is the unintentional impact on the microstructure when the samples are prepared for analysis. To visualize the inner pore structure, you have to cut the pellet or even make very thin slices. This will – even if you freeze the pellet or use very sharp knifes – affect the structure and potentially influence the interpretation of the results. Instead, X-ray microtomography (µCT) (also described by Draganovic *et al.*, 2013) is preferred as it is non-destructive. Thereby, you can analyse an entire pellet, from core to surface, without affecting its structure and cutting it into crumbles. The read-out is exported as a 3D structure and allows for specific analyses of interest, *i.e.* pore volumes, pore size distributions, and pore surface areas.

The specific µCT analyses were carried out at Danish Technological Institute using a SkyScan 1172 X-ray microtomography scanner (Bruker microCT, Kontich, Belgium) mounted with a Hamamatsu C9300 (Naka-ku, Japan) 11 megapixel CCD camera. The pixel size was set to 8.8 µm, while the voltage and current were adjusted to 59 kV and 167 µA, respectively. The image data were computed by the SkyScan software CTAn v.1.13.2.1. using the Multilevel Otsu method at 4 threshold levels for optimal channel adjustment.

5.5. Oxidation

In BioMar, oxidative stability is measured in hours using an instrument known as Oxipres (Mikrolab Aarhus A/S, Højbjerg, Denmark). A sample of fish feed pellets estimated by in-house NMR to contain 5 g of oil is loaded into the instrument, flushed by and successively exposed to 5 bar of pure oxygen in an airtight pressure chamber. The chamber is heated to 80 °C to – along with the oxygen atmosphere – accelerate the lipid oxidation. The pressure inside the chamber is constantly monitored and will initially decrease slowly while the antioxidants in the feed protect the lipids against oxidation. When there are no more antioxidants available, the pressure will decrease faster. This sudden change in oxidation speed – visualized as a sudden change in the pressure inside the chamber – is interpreted as the shelf life and has to be above 100 hours to fulfil the internal quality criterion.

5.6. Viscosity

To understand and describe the flow dynamics of the dough in the die section, it is necessary to know the dough viscosity. To get consistent and representative measurements, it is necessary to perform the rheological tests under extrusion conditions, *i.e.* pressure above the atmosphere, low water inclusion, and temperatures above 100 °C. By means of a slit-die rheometer at Karlsruher Institut für Technologie (KIT), Germany, the viscosity of different recipes was measured under realistic extrusion conditions. The setup included three pairs of pressure sensors along the slit-die (*fig. 11*). For further details regarding instruments and methods, reference is made to Horvat *et al.* (2013). The viscosity was insignificantly affected by the composition of the five different mealmixes tested at KIT, and was finally defined by a power law exponent, n, of 0.20, and a consistency index, m, of 450 Pa·s^n. This is very much in accordance with Lam & Flores (2003), cf. *4.2. Nucleation*.

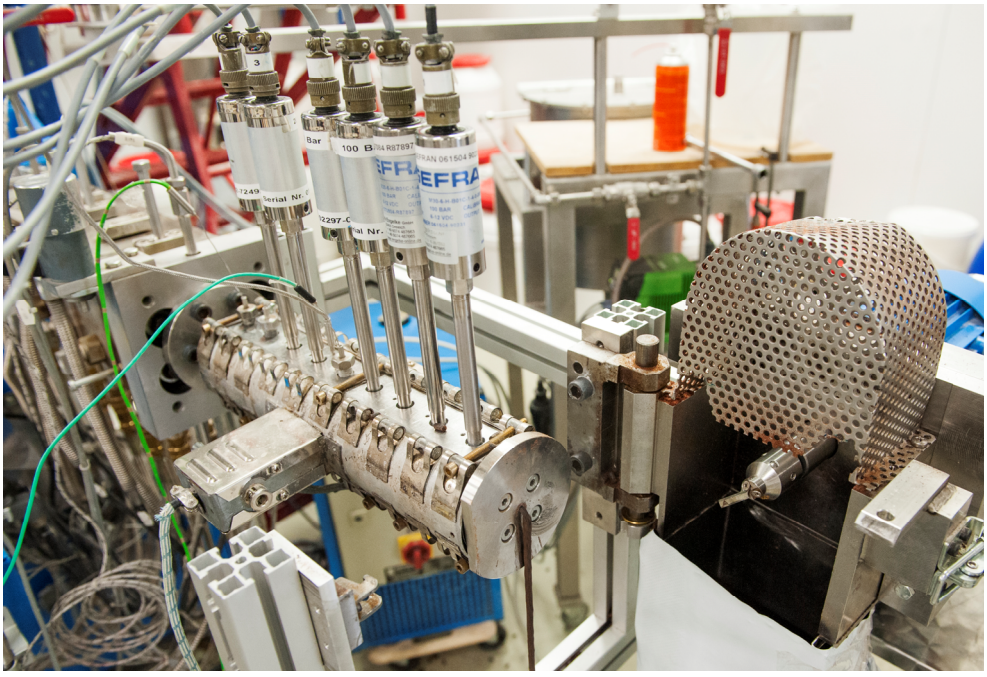

Fig. 11
Slit-die rheometer mounted with three pairs of pressure sensors at KIT.

To illustrate the prevailing flow behaviour, turbulent or laminar, cf. *4.3. Pellet Shape*, *equation 4* is used to calculate Reynolds number using the input parameters from the power law model. The mean velocity, $\bar{v}$, is most often – depending on many different factors, *e.g.* pellet size, capacity, extruder, die plate – in the range of 1-1.5 m/s throughout the individual die sections. In reality, the Reynolds number is not that sensitive to changes in the diameter, *D*, of the die exit, as the number of holes per die unit increases when *D* decreases. The density, ρ, of the dough just before exit is in the range of 1200-1250 kg/m^3:

$$Re = \frac{8 \cdot \bar{v}^{(2-n)} \cdot \left(\frac{D}{2}\right)^n \cdot \rho}{m \cdot \left(\frac{3 \cdot n+1}{n}\right)^n} \qquad (4) \qquad \textit{[based on Risum \& Friis (2009)]}$$

Equation 5 is used to calculate the critical Reynolds number, Re_c, to determine the transition from laminar ($Re < Re_c$) to turbulent flow ($Re > Re_c$):

$$Re_c = 2100 \cdot \frac{(4n+2) \cdot (5n+3)}{3 \cdot (3n+1)^2} \qquad (5) \qquad \textit{[based on Risum \& Friis (2009)]}$$

When $n = 0.20$, Re_c is $3.1 \cdot 10^3$.

It is easy to realise – even when inserting extreme values in *equation 4* – that *Re* in the linear die section will never be above Re_c. More likely, *Re* will be in the range of 5-20. Consequently, it is unrealistic to obtain a true turbulent flow, and other auxiliary means, *e.g.* increased shear or flow disturbance, are needed to interrupt a stable translational flow creating elongated and parallel pores, cf. *4.2. Nucleation*.

6. Practical Use

At this stage, enough theories and methods have been presented to venture into development of new concepts and inventions. Firstly, a fish feed pellet will be observed as an individual system to illustrate its current functionality, then the pellet structure will be utilized for delivery of components, and lastly, the structure will be optimized to provide its optimal physical pellet quality.

6.1. Extrudate as Delivery System

Inspired by respectively Singh *et al.* 2010 and Boon *et al.* (2010), studying microparticles as drug delivery systems and functional foods for delivery of carotenoids, the idea of perceiving the extrudate, *i.e.* a fish feed pellet, as a similar system was developed. Most of the nutritional energy as well as the heat-labile additives, cf. *4.8. Oil Leakage*, is added during coating. Hence, it is reasonable to describe a fish feed pellet analogous to a medical pill: The capsule is simply a drug reservoir to protect and transport the API (Active Pharmaceutical Ingredient) in the same way as the extrudate structure transports a large fraction of the nutrients from the factory to the fish cages. The advantage of using the extrudate as a spacious capsule, moving more of the nutrients to the coating step, is that the heat-sensitive raw materials can be handled more carefully. However, this approach makes even higher demands on the physical quality of the extrudate and requires more knowledge regarding the location and behaviour of the coating inside the pellet structure.

6.2. Coating

As the reader is most likely already familiar with coating technology, this paragraph is more serving as a link to the published paper and how it created the foundation for the filed patent applications. For details about the paper, reference is made to Dethlefsen *et al.* (2016) (enclosed in the public thesis, whereas the patent applications are only enclosed in the confidential thesis).

The uncoated extrudate consists of processed raw materials, constituting the pellet structure, and pores full of air. The air-to-material[13] ratio before pellet coating is, depending on recipe and required sinking properties, about 1:1. During coating, the dry pellets are exposed to approx. 80% vacuum. The coating mixture is then added to the surface of the pellets during slow release of the vacuum and constant agitation. Hereby, the coating is effectively transported from the surface into the open pellet structure.

Many different coating methods are applied across the factories, covering different vacuum levels, release times, and separate steps of coating addition, *e.g.* adding rape seed oil with low viscosity in the beginning of the vacuum release, and later on adding fish oil with higher viscosity. Even combining the last coating step with hardened oils to minimize oil leakage. The term *coating* is used when the entire extrudate structure is treated, whereas top-coating refers to the surface only.

[13] ”material” in the meaning of the processed raw materials constituting the pellet structure.

During a storage experiment of astaxanthin-coated fish feed pellets, it was clear that the core of the pellet structure had the ability to protect the oxidative unstable additive (*Paper I*: Dethlefsen *et al.*, 2016). Additionally, it was demonstrated that an improved utilization of the pores had the potential to improve the stability of extruded fish feed. Having realized the potential of the unused pores, a creative process of utilizing the pellet structure in a smarter and more optimal way was initialized. This process led to the filing of two patent applications:

Patent I: Method and device for producing a fish feed containing probiotic bacteria
Patent II: Nozzle for an extruder

In the following, the sequence of thoughts and observations governing the patents will be presented. First, regarding *Patent I*, how the protective pellet core is used to shield probiotic bacteria added during coating, and second, regarding *Patent II*, how the pellet structure can be improved by means of a novel die design. Due to the confidentiality of the patent applications, there will be no detailed description of these applications in the next paragraphs. For details, reference is made to the patent applications enclosed in the confidential thesis. As the public thesis has to be sufficient, it will contain all necessary details as well, however, sensitive information is anonymized.

6.3. The Bactocell Challenge

To achieve better fish health and growth, probiotics are added to increasingly more BioMar recipes. The specific bacterium is *Pediococcus acidilactici*, internally most often referred to as Bactocell. As described under *4.8. Oil Leakage*, probiotics are, due to their sensitiveness to high temperature, added during coating. Focusing on the *Z*-value in relation to the decimal reduction time, *D*, bacteria in general are much more sensitive to changes in temperature than enzymes, pigments and vitamins (Awuah *et al.*, 2007). Consequently, the addition of bacteria is correspondingly more critical to the temperature of the oil and pellets. After drying and prior to coating, the pellets are most likely above 65 °C, and even if more gentle drying conditions are used, the temperature of the pellets is still above 50 °C when the oil is added during coating. If Bactocell is exposed to this high temperature, 70-80% of the applied bacteria are inactivated in the coating process. Of course, a two-step coating could be utilized, first applying cold oil to cool the pellets, and then applying the bacteria-containing oil on the top. However, the low temperature of the oil from the first coating step would increase the oil viscosity and reduce the amount of possible oil to add during coating. Also, the bacteria would primarily be located on the surface of the pellets, and consequently be more exposed to wash-off and degradation compared to bacteria protected in the pellet core, cf. *6.2. Coating*.

To solve this task, we performed different coating experiments to explore the effects of oil and pellet temperature, pressure, and process aids on the survival of Bactocell. The pellets were analysed at Lallemand Blagnac laboratory, France, by CFU count (Colony Forming Units) and the method was reported to have an accuracy of ±0.5 log. The most consistent results, leading to more

than 80% survival of Bactocell, were obtained when coating the pellets with a mixture of oil, Bactocell and a process aid, having an equilibrium temperature below 30 °C. The pellets should have as low temperature as possible, but most likely about 55 °C. The initial coating pressure should preferably be below 50 mbar, corresponding to 95% vacuum. Hereby, the combination of temperature, humidity and pressure in the vacuum chamber, allows for evaporative cooling which was shown to be sufficient to protect the probiotic bacterium against unacceptable lethal reduction. Reference is made to *Patent I* for more details regarding procedure and process aids.

6.4. Pore Investigation

From the theory given in *chapter 4* and the practical use of the extrudate structure to protect and deliver additives presented in *paragraph 6.1-6.3*, the pores are clearly of interest when optimizing the physical pellet quality. The two obvious questions are, cf. *fig. 1*:

1. What is the optimal pore structure?
2. How to obtain the optimal pore structure?

First, to investigate the optimal pore structure from an oil leakage perspective, it is reasonable to investigate a fish feed pellet during the steps involved in the single pellet coating test. A group of fish feed pellets were – according to the SPC test described in *5.3. Oil Leakage* – captured by µCT before and after single pellet coating, as well as after centrifugation. The SPC and image alignment made it possible to track the individual pellets and even compare the exact same cross sections of the pellet at all three stages of the SPC test. Hereby, it is possible to infer which pores the oil is leaking from. An example of a pellet captured during SPC is shown in *fig. 12*. The squares show relevant large pores, whereas the circles show relevant small pores. It is clear that the oil is primarily leaking from the large pores, but is retained in the small pores even after centrifugation for 5 minutes at 50 *g*. Additionally, by comparing the surfaces of mainly the oil-empty pores before coating (*fig. 12a*) and after centrifugation (*fig. 12c*), it is obvious that even on the surface of the large empty pores, a thin layer of oil appears to be retained in the end of the SPC test. Thus, it is reasonable to suggest that a large pore surface area is preferred to minimize oil leakage.

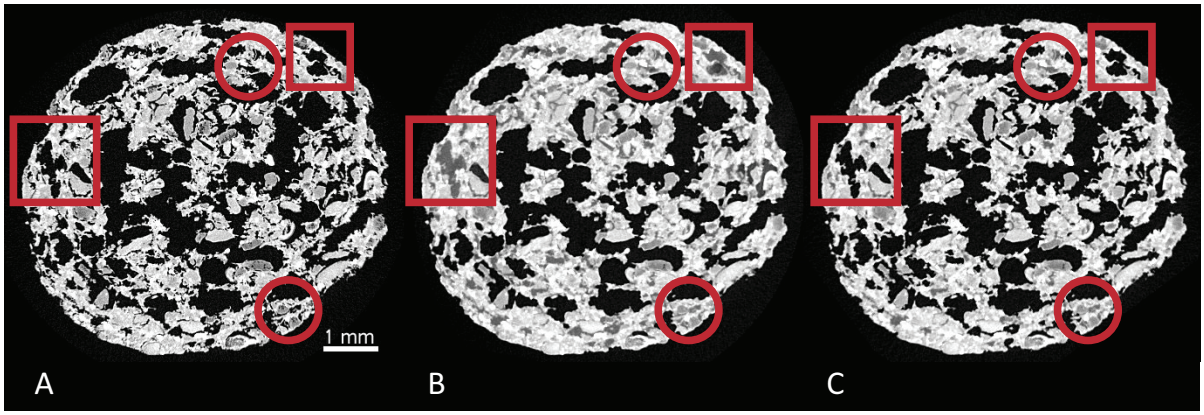

Fig. 12
Cross section of fish feed pellet before coating (A), after coating (B), and after centrifugation (C). The pellet has been treated according to the SPC method described in *5.3. Oil Leakage*. The squares show relevant large pores, whereas the circles show relevant small pores. It is clear that the oil is primarily leaking from the large pores, but is retained in the small pores even after centrifugation. Black is air, light grey is pellet structure, and dark grey is oil.

To investigate the validity of this statement, nine batches of fish feed pellets were produced using three different dies. Only one recipe was used for all productions, and everything – besides the die design, grinding of the meal mix, and screw elements in the last barrel section – was kept constant. With reference to *fig. 13*, series 1, 2, and 3 had 1 mm, 0.5 mm, and 1 mm of grinding, respectively, whereas only series 3 had dedicated mixing elements in the last section of the barrel. The diameter of the exit was 6 mm for all three dies; however, the die design prior to the land section differed. For details regarding specific design of Die A-C, reference is made to *Appendix 1* in the confidential thesis. Inspired by Draganovic *et al.* (2013) using different recipes to change the microstructure of the extrudate, I tried to obtain different microstructures without changing the recipe. From *fig. 13*, it is clear that the axial and radial expansion were very different just by shifting from one die to another. Also, the pore structure was highly affected by the die design. Be aware that the presented slices are not necessarily representative for the entire batch, and not even the individual pellet, as each pellet scan consists of more than 2,000 pictures. Next, four pellets from each batch were treated by SPC and captured by µCT. The amount of oil added during coating and the remaining oil retained in the fish feed pellets were plotted against the pore volume and the pore surface area of the fish feed pellets, respectively, and the correlations are presented in *fig. 14-15*. In total, 36 pellets were single pellet coated and captured by µCT, but one pellet from trial B1, cf. *fig. 13*, was removed as an outlier, leaving 35 pellets for the investigation of the pores' impact on oil leakage. The outlier is indicated by a red square in *fig. 14-15*. Only in *fig. 14*, it is clear that the pellet is an outlier, whereas the sample fits the overall trend in *fig. 15*. This is in accordance with the observation noted during SPC of the specific sample, *i.e.* floating in the oil during coating, which indicates empty pores. From the appearance of the sample in *fig. 15*, the surface of the pores seems however to be covered with oil, even though the pores are not full of oil. This supports the observation regarding a thin layer of oil on the surface of the pores in *fig. 12C*.

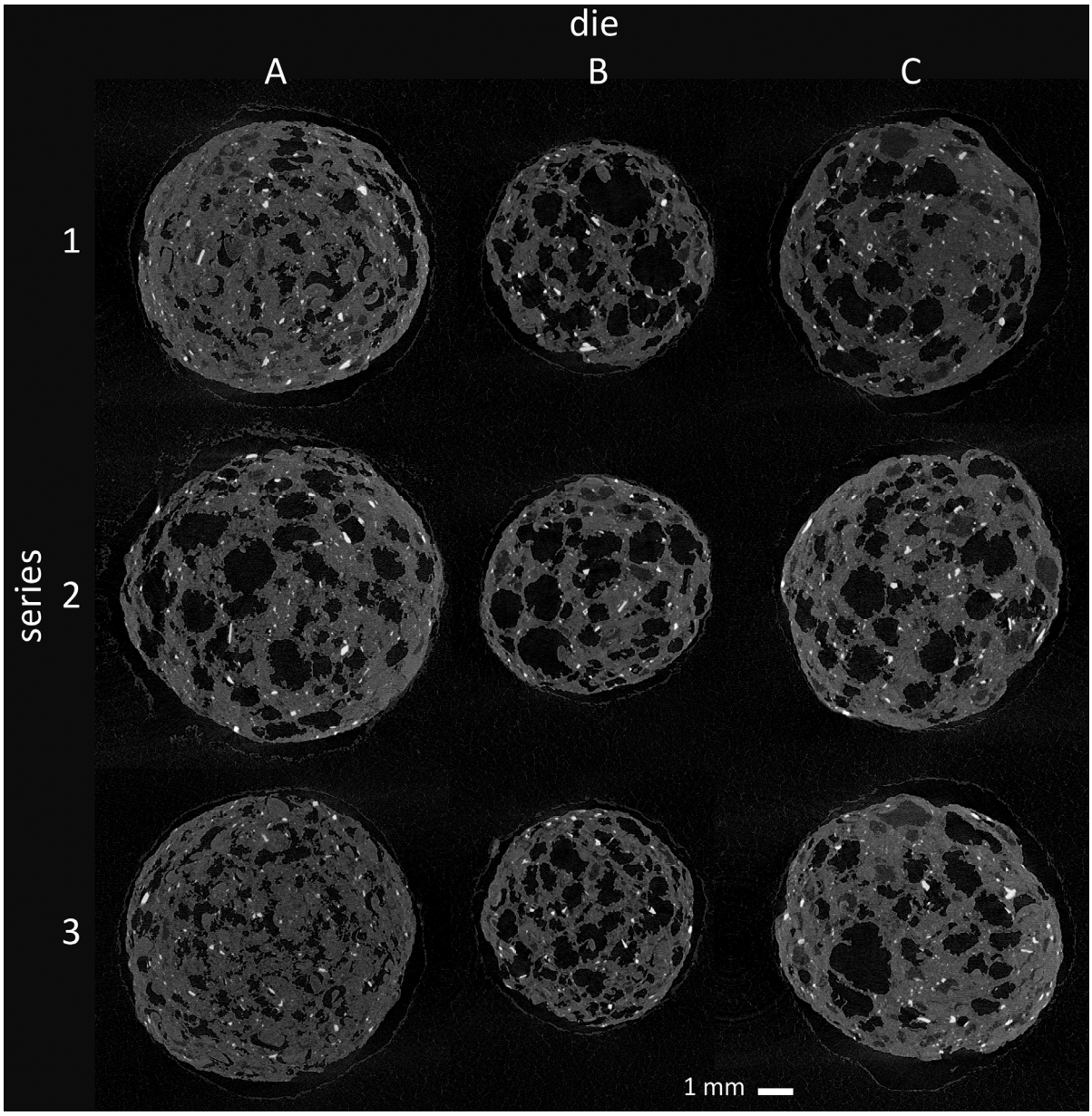

Fig. 13
µCT images of nine SPC treated fish feed pellets. Black is air, white is bone fragments, light grey is pellet structure, and dark grey is oil residues. Three different dies were tested (A, B, C) using the same recipe but different grinding of the meal mix prior to extrusion and degree of mixing using different screw elements in the last barrel section. Row 1 had 1 mm grinding and no dedicated mixing elements, row 2 had 0.5 mm grinding and no dedicated mixing elements, row 3 had 1 mm grinding and dedicated mixing elements. The diameter of the exit was 6 mm for all three dies; however, the die design prior to the land section differed. For details regarding specific design of Die A-C, reference is made to *Appendix 1* in the confidential thesis.

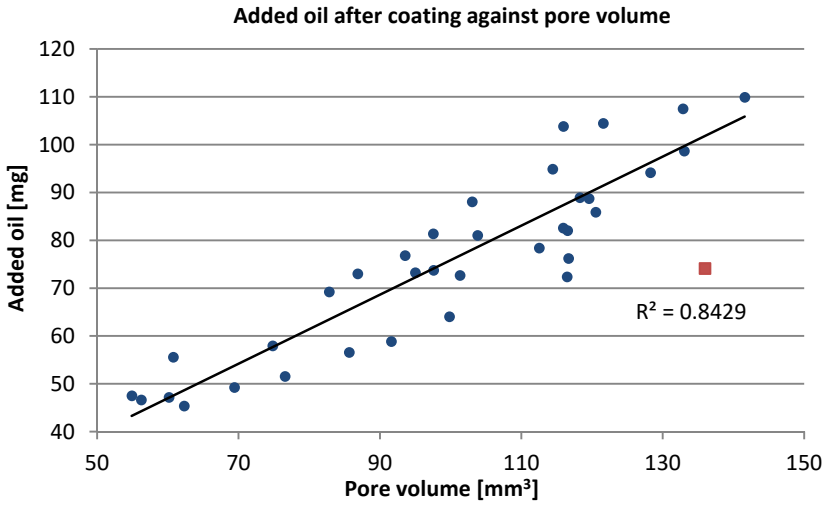

Fig. 14
Amount of added oil during single pellet coating of 35 fish feed pellets plotted against the pore volume of the respective pellets. The pellets are coated by SPC, and the pores are investigated by µCT. The outlier is indicated by the red square.

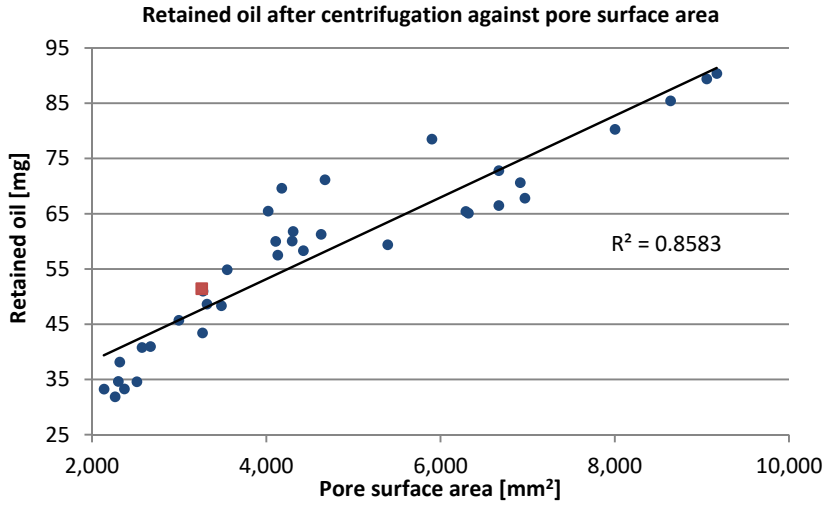

Fig. 15
Amount of retained oil after centrifugation of 35 fish feed pellets plotted against the pore surface area of the respective pellets. The pellets are coated by SPC, and the pores are investigated by µCT. The outlier from *fig. 14* is indicated by the red square.

From evaluation of *fig. 12* and *fig. 14-15*, it is possible to make a conclusion concerning the optimal pore structure to reduce oil leakage: While the amount of oil possible to add to a fish feed pellet correlates to the volume of the pores, the amount of retained oil after centrifugation correlates to the total surface area of said pores. To obtain the optimal pore structure, the volume of the pores should ideally be distributed among many small pores to give the highest pore-surface-area to object-volume ratio (S:V ratio). The next paragraph aims to provide a better understanding of the impact of the die design on the S:V ratio of extruded fish feed pellets.

6.5. Educated Pursuit of Next Generation Dies

To test different principles inspired by the theory presented in *chapter 4*, three new types of dies (Die E-G) were designed and 3D printed with nylon SLS (Selective Laser Sintering). Also, a standard die was printed as control (Die D). The exit diameter of all dies was 6.5 mm. For details regarding specific design of Die D-G, reference is made to *Appendix 2* in the confidential thesis. The recipe and extruder settings were kept constant during the die comparison trial. Two of each die was used simultaneously, and the feeding rate was set to 150 kg/h on the pilot plant twin screw extruder BC45 (Clextral, Firminy, France). Eight pellets from each batch were treated by SPC, and four of the eight pellets per batch were additionally captured by µCT. The fraction of oil in the pellets after single pellet coating and after centrifugation are shown in *fig. 16*, whereas the S:V ratio results from the µCT captured pellets are shown in *fig. 17*. From *fig. 16*, it is seen that it is possible to achieve a significantly higher oil content in pellets produced using Die G compared to pellets produced using Die E and Die F. Also, pellets produced using Die G contain a significantly higher oil content after centrifugation compared to pellets produced using Die E. Similarly, from *fig. 17*, pellets produced using Die G have a significantly higher S:V ratio compared to pellets produced using Die D and Die E. Likewise, pellets produced using Die F have a significantly higher S:V ratio compared to pellets produced using Die E.

From the enhanced understanding of the pores' impact presented in *6.4. Pore Investigation* and the findings summarized in *fig. 16-17* in the present paragraph, Die G, and partly Die F, is of particular interest to test in full scale production. Both of these dies utilize the ideas regarding shear and flow disturbance introduced in *chapter 4* and paragraph *5.6. Viscosity*. Hence, the theory and practice agree when testing in small scale production. Next step is to gain an even better understanding of the flow behaviour in Die D and Die G by utilizing Computational Fluid Dynamics (CFD). As the basic principles and mechanisms have already been presented, reviewed and validated, the next generation dies to be tested in full scale production will primarily be evaluated by SPC, partly as a result of economic considerations, cf. *1.5. Results*, partly because the SPC is directly providing the results of interest, *i.e.* extent of oil leakage.

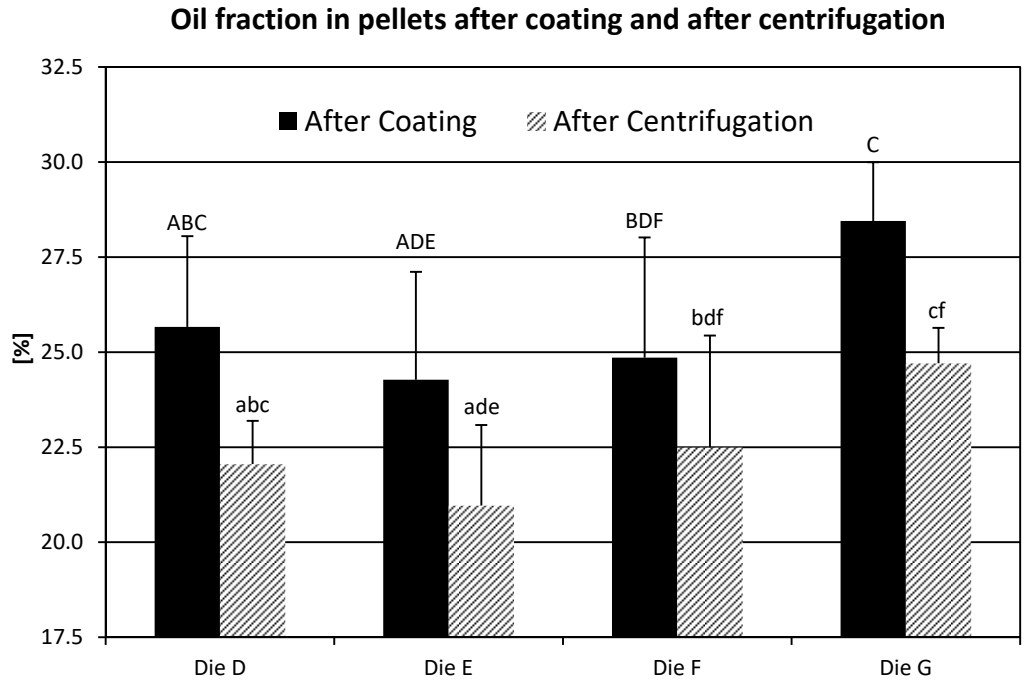

Fig. 16
Oil fraction in fish feed pellets measured by SPC. The pellets were produced using four different dies. The recipe and extruder settings were kept constant. The exit diameter of the dies was 6.5 mm. For details regarding specific design of Die D-G, reference is made to *Appendix 2* in the confidential thesis (Die D was the control with a standard design). Eight pellets per batch were treated by SPC. The total fat content in the fish feed pellets is higher than the presented figures as only the oil added during coating is included in the balance. After coating: Pellets from Die E are significantly different from Die G, and pellets from Die F are significantly different from Die G. After centrifugation: Pellets from Die E are significantly different from Die G.

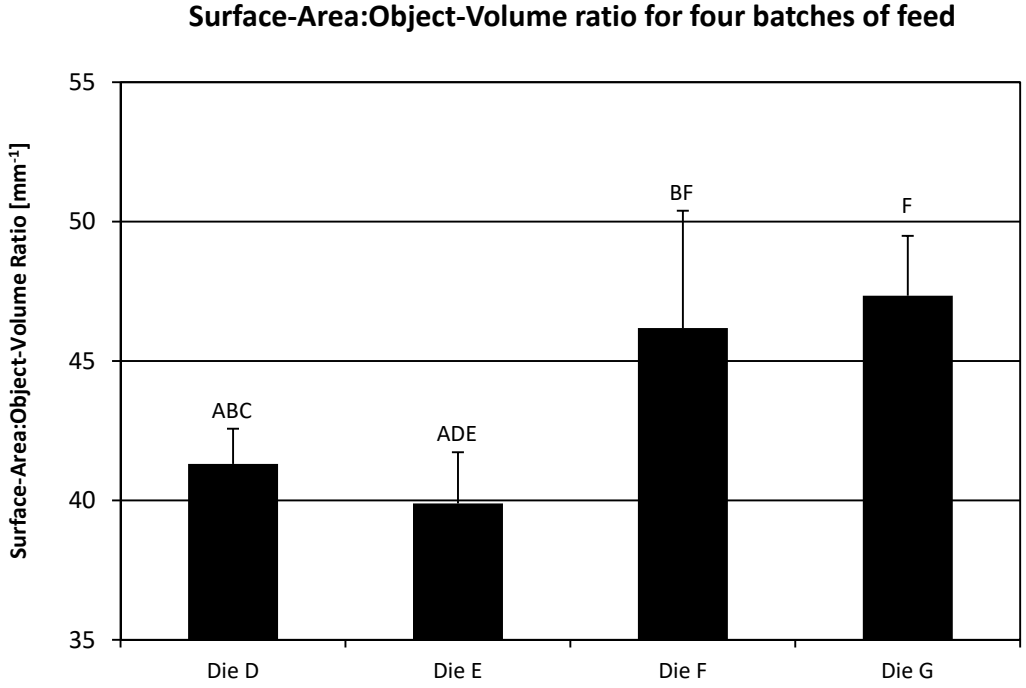

Fig. 17
Four of the pellets from each batch presented in *fig. 16* were captured by µCT, and the pore surface area and total volume of pellet material were measured for the individual samples. Then, the surface density was calculated for each pellet and expressed as the surface-area:object volume (S:V) ratio. Pellets from Die D are significantly different from Die G, and pellets from Die E are significantly different from Die F and Die G.

6.6. Simulating the Flow through the Dies

Die G has been identified as the most promising design to test in full scale at the Joint Venture BioMar SA factory in Pargua, Chile, and at the factory in Brande, Denmark. Die G and the corresponding control die (Die D) have been designed for the production of 7-8 mm pellets at the pilot plant extruder running at 150 kg/h. The productions in Chile and Brande are demanding 12 mm and 10 mm pellets, respectively, and capacities at around 12,000 kg/h wherefore upscaling of the dies is necessary. However, it is rarely possible to just scale up process equipment and then expect the physics to be unaffected. Therefore, before going full scale, the flow through the dies was simulated using the CFD module in COMSOL Multiphysics® Modeling Software v.5.2a (COMSOL, Inc., Massachusetts, USA). *Fig. 18* shows the dimensions of the upscaled control die (Die D*) adjusted to fit the die plate in Brande. The green vertical lines indicate the three cross sections marked with dashed white lines in *fig. 19*. The location of these cross sections is identical for the corresponding upscaled Die G (designated as Die G*) which – along with the respective simulations – is available in *Appendix 3-4* in the confidential thesis.

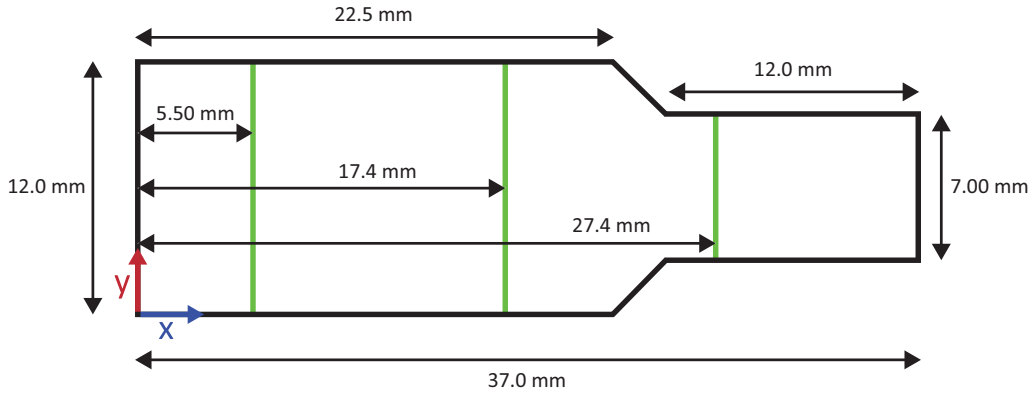

Fig. 18
Illustration of the control die (Die D*) designed to fit the die plate in Brande, Denmark. The die is intended for 10 mm pellets. The green vertical lines indicate the three cross sections marked with dashed white lines in *fig. 19*. The location of these cross sections is identical for the corresponding upscaled Die G (designated as Die G*). The flow direction follows the x co-ordinate.

In addition to the die dimensions from *fig. 18* and the rheological properties presented in *5.6. Viscosity*, the COMSOL model was as well loaded with the following thermophysical values for the dough[14]:

- heat capacity, c_p: 2,490 J/kg/K
- density, ρ: 1,227 kg/m^3
- thermal conductivity, k: 0.391 W/m/K

Also, the following simplifications were necessary due to computational limitations, *i.e.* mesh sensitivity and processing power:

[14] These values are theoretical and calculated according to Risum & Friis (2009) using a standard BioMar recipe.

- The model includes no phase shift of water, and the thermophysical properties of the dough are constant throughout the die.
- The die is only simulated in 2D. However, the dies are either fully or partly symmetrical along the x co-ordinate.

The three simulations of interest are shown in *fig. 19*, covering flow velocity (A), shear rate (B), and apparent viscosity (C). The three dashed vertical white lines in *fig. 19A-C* correspond to the graphically presented cross sections (1-3) available in *fig. 20-22*, respectively.

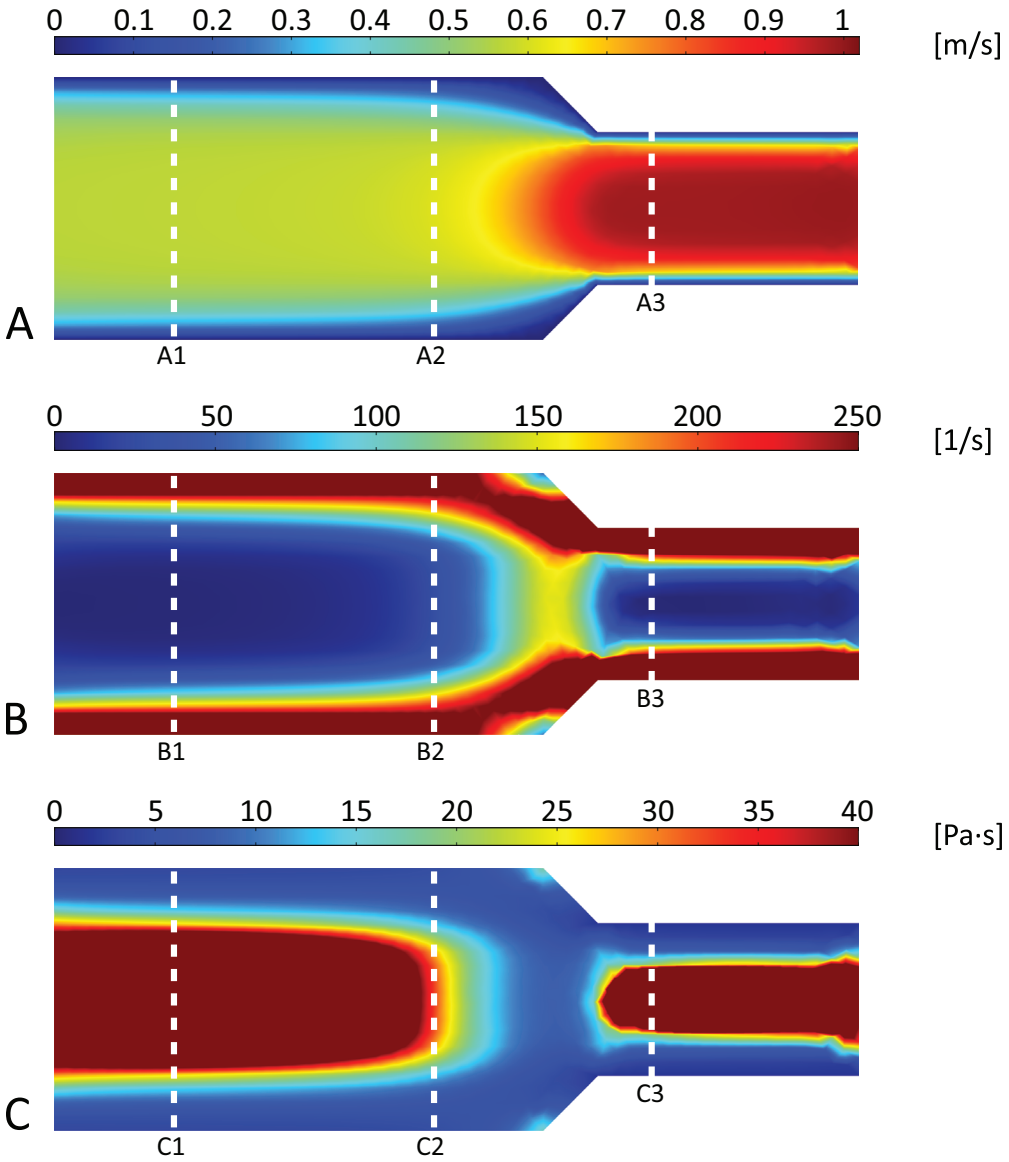

Fig. 19
Simulations of interest for Die D* covering flow velocity (A), shear rate (B), and apparent viscosity (C). The three dashed vertical white lines in each partial simulation correspond to the graphically presented cross sections (1-3) available in *fig. 20-22*. Reference is made to *Appendix 3* for the corresponding simulations for Die G*. The flow direction follows the x co-ordinate shown in *fig. 18*.

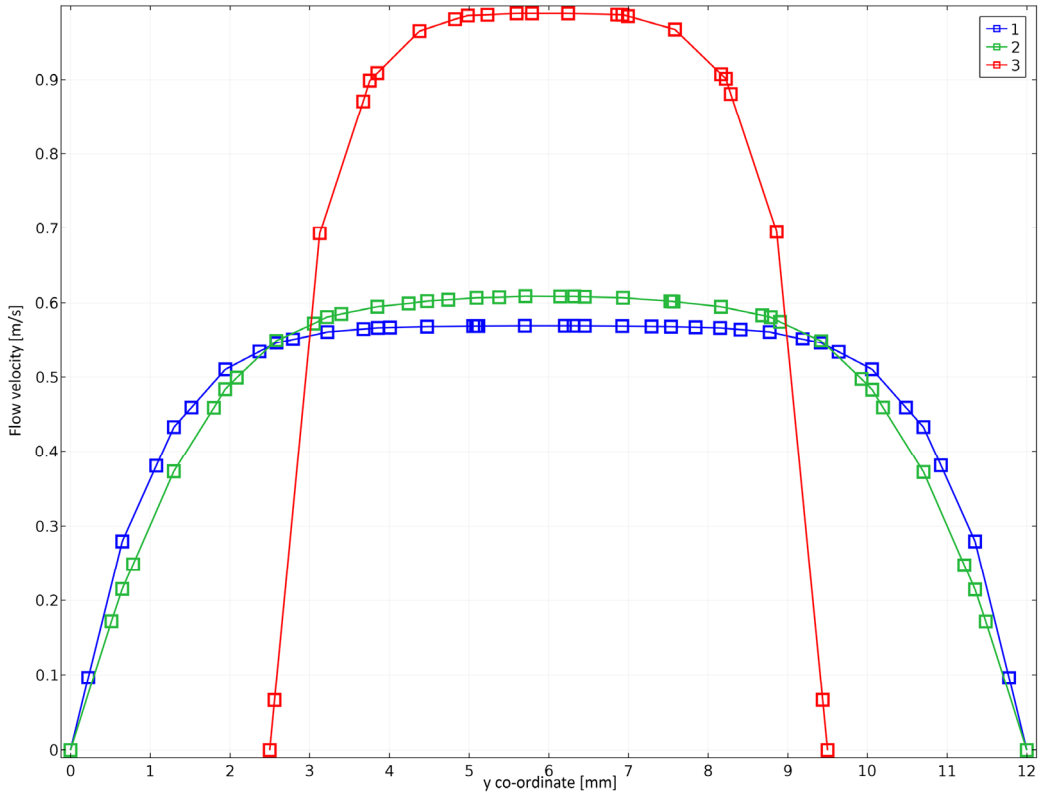

Fig. 20
Flow velocity profile 1-3 for cross section *A1-A3*, respectively, cf. *fig. 19A*. Reference is made to *fig. 18* for details regarding orientation of the co-ordinate system. The squares are simulated using COMSOL, and then interconnected to improve readability. Reference is made to *Appendix 4* for the corresponding Die G*-simulation.

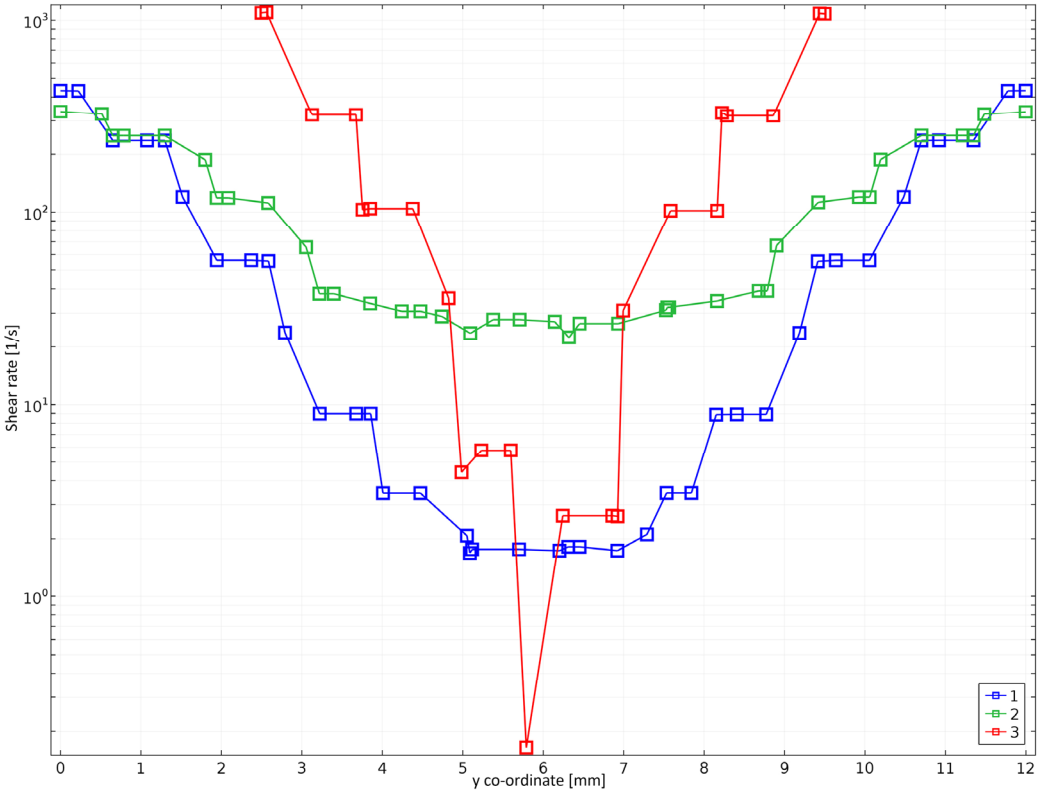

Fig. 21
Shear rate profile 1-3 for cross section *B1-B3*, respectively, cf. *fig. 19B*. Reference is made to *fig. 18* for details regarding orientation of the co-ordinate system. The squares are simulated using COMSOL, and then interconnected to improve readability. Reference is made to *Appendix 4* for the corresponding Die G*-simulation.

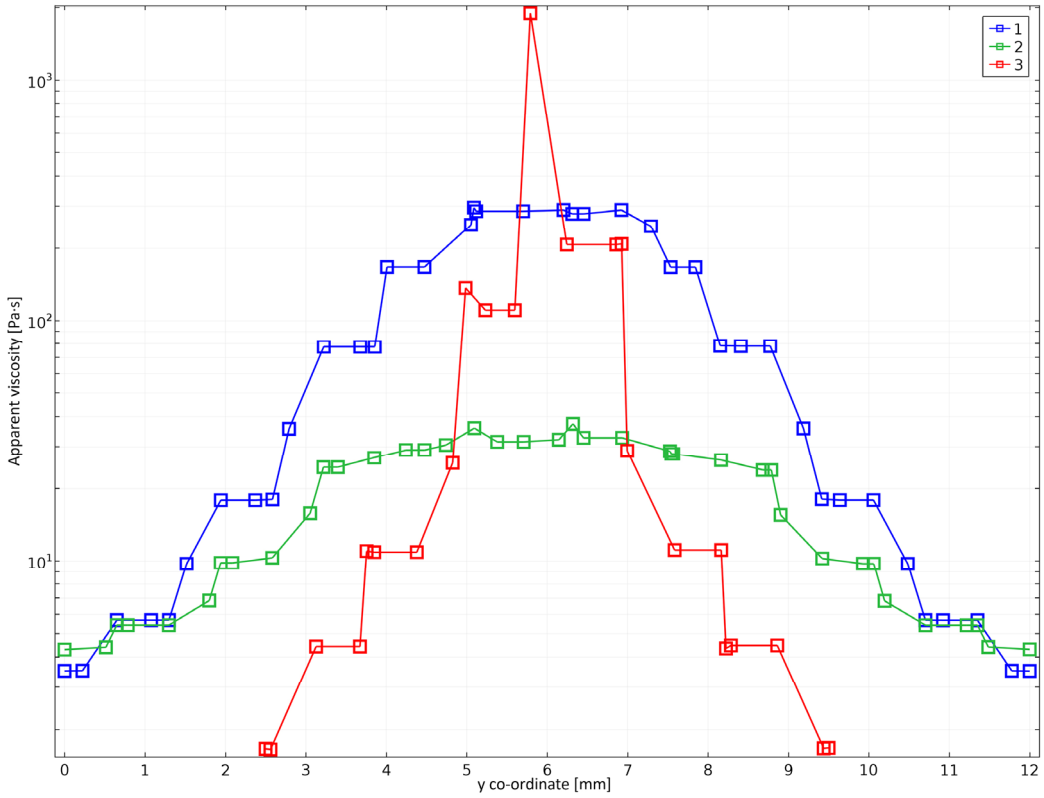

Fig. 22
Apparent viscosity profile 1-3 for cross section *C1-C3*, respectively, cf. *fig. 19C*. Reference is made to *fig. 18* for details regarding orientation of the co-ordinate system. The squares are simulated using COMSOL, and then interconnected to improve readability. Reference is made to *Appendix 4* for the corresponding Die G*-simulation.

The die design in *fig. 18* is relatively simple as it consists of only two symmetrical pipes in line, and the simulations presented in *fig. 19* are very much in accordance with the expectations in consideration of *5.6. Viscosity*. In theory, the flow velocity of the dough is 0 m/s at an infinitesimally thin boundary layer between the inner surface of the die and the dough (Risum & Friis, 2009). Additionally, the present flow is characterized as laminar. Consequently, even though the flow velocity at the boundary layer is low, cf. *fig. 19A*, the highest velocity gradient is present close to the die surface (Risum & Friis, 2009). This is confirmed by the gradients of the flow velocity profiles in *fig. 20*.

Fig. 19C is almost an inverted version of *fig. 19B* – and this makes sense, considering *equation 1* in *4.2. Nucleation*, proclaiming the apparent viscosity to be negatively correlated to the shear rate in cases where the power law exponent is below 1. The variation in apparent viscosity across the die is shown in *fig. 22*. At cross section C3, cf. *fig. 19C*, the apparent viscosity in the centre of the die is about 2 log units larger than close to the surface. This gradient in apparent viscosity causes the centre of the pellet structure to solidify in the land section of the die, thereby decreasing further pore formation in the core region, cf. *4.2. Nucleation*. The corresponding simulations for Die G* are available in *Appendix 3-4* in the confidential thesis. As the volumetric flow rate is identical for the Die D* and Die G*, the mean linear flow velocity of the dough is also the same for both dies. However, the maximum linear flow velocity in the land section is about 25% higher in Die D* than Die G*, indicating a more laminar and parabolic flow profile in Die D* (Risum & Friis, 2009).

6.7. Full Scale Production in Chile and Denmark

After the CFD simulations of the Die D* and Die G*, the dies were manufactured in PEEK and PEEK with inserts of 3D printed SLS, respectively. The simulations derive from the dies tested in Brande, Denmark, and were intended for 10 mm pellets, whereas the dies tested in Pargua, Chile, were slightly bigger and intended for 12 mm pellets. Also, the recipes were different at the two factories, but kept constant during each comparative test. As the aim of the full scale trials is to compare the physical quality of pellets produced using a standard die against a novel design, it is without importance that the setup in Chile and Denmark is not identical as long as the major process conditions at the respective factories are kept constant during the comparative tests. The details of primary interest are listed in *table 2*.

Table 2, Overview of specific settings and conditions present during the trials in Chile and Denmark for the comparison of Die D* and Die G*.

Factory	**BioMar SA JV, Pargua, Chile**		**BioMar A/S, Brande, Denmark**	
Die design	**Die D***	**Die G***	**Die D***	**Die G***
Capacity [kg/h]	12,000	12,000	12,000	10,000
Pressure at die [bar]	30	37	25.4	42.4
Ø, die exit [mm]	8.4		7.0	
Number of dies	28		48	
Open area of dies [cm^2]	16		18	
Bulk density [kg/m^3]	455	454	444	440
Extruder type	Single screw		Twin screw	
Extruder model	Andritz EX1250		Clextral BC160	

The most striking difference in conditions present during the individual factory trials is the pressure at the dies. In both cases, the pressure is higher for Die G*. Even when the capacity is lowered, as in Denmark, the pressure is still higher. The primary reason for the much higher pressure for Die G* in Denmark is due to a reduced open area in the dies prior to the land section. The same die narrowing is present for Die G* in Chile, however, the larger open area in the Chilean die's land section reduces the extent of the pressure increase.

Even though the in-house laboratories in Chile and Brande are following the same test methods, they are not providing the same level of details for the measurements. This emphasizes the necessity of my own analyses performed at DTU, *i.e.* oil leakage using SPC and pellet strength by TA. *Table 3* provides an overview of the results from Chile and Denmark with the level of details provided by the respective laboratories.

Table 3, Overview of the results provided by the in-house laboratories in Chile and Denmark for the analyses of pellets produced at the respective factories during the trials comparing Die D* and Die G*.				
Factory	**BioMar SA JV, Pargua, Chile**		**BioMar A/S, Brande, Denmark**	
Die design	**Die D***	**Die G***	**Die D***	**Die G***
SF [%]	3.3	0.74	0.35	0.080
Doris [%]	78.3	78.6	73.7	87.2
Kahl [kg]	10.8 ± 1.49	8.30 ± 1.11	-	-
TVT [N]	-	-	52	55

If the results presented in *table 3* were the only available, it would be impossible to conclude anything. Even though it has been possible to provide the Kahl values from Chile with a standard deviation – and the two groups are significantly different – the reliability of the test method is questionable, cf. *5.1. Strength*. Fortunately, more statistics are available from my own analyses, and the results are presented in *fig. 23-27*. The reason for the lack of texture analyses for the Chilean pellets is due to a national strike in Chile related to an algal bloom crisis. The strike delayed the delivery of the pellets to Denmark more than four months, making any further testing of their physical strength pointless as microcracks and general weakening of the pellet structure are likely to happen even within the first three weeks after production (Wolska, 2014).

The oil leakage for the Chilean pellets measured by SPC is significantly smaller for the pellets produced using Die G* than for Die D*, cf. *fig. 23*. However, the amount of oil added during the analysis is also significantly smaller for Die G* than for Die D*. Therefore, the reason for the corresponding lower loss of oil could as well be the result of the lower oil content after coating in the pellets produced by Die G*. Nevertheless, lower oil leakage (SF) for Die G* was, cf. *table 3*, reported by the Chilean factory as well, and Production Chief, Johny Cabezas, reported that they had never previously managed to produce such oil-dense diet with that low oil leakage as they experienced using Die G*.

For the Danish trial, the SPC is more representative as there is no significant difference between the two groups of pellets regarding the fraction of oil added to the pellets during coating, cf. *fig. 24*. After centrifugation, the fraction of oil is significantly higher for the pellets produced using Die G* than for Die D*, and the corresponding oil loss is significantly smaller for the pellets produced using Die G* than for Die D*. This is in agreement with the SF measurements presented in *table 3*.

Results regarding the physical strength measured with by TA in accordance with *5.1. Strength* are presented in direct continuation of the SPC results.

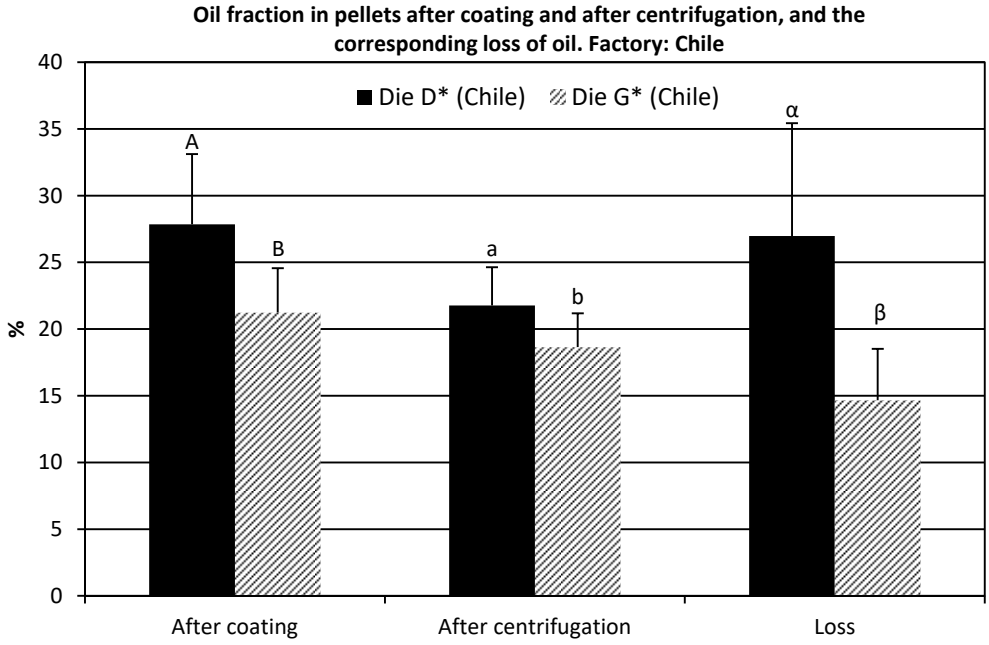

Fig. 23

Results from SPC of pellets from the Chilean factory produced using Die D* and Die G*. Eight pellets from each group were treated. The pellets from Die D* contain significantly more oil after coating and after centrifugation than the pellets produced using Die G*. However, the loss of oil during centrifugation is significantly smaller for Die G* than for Die D*.

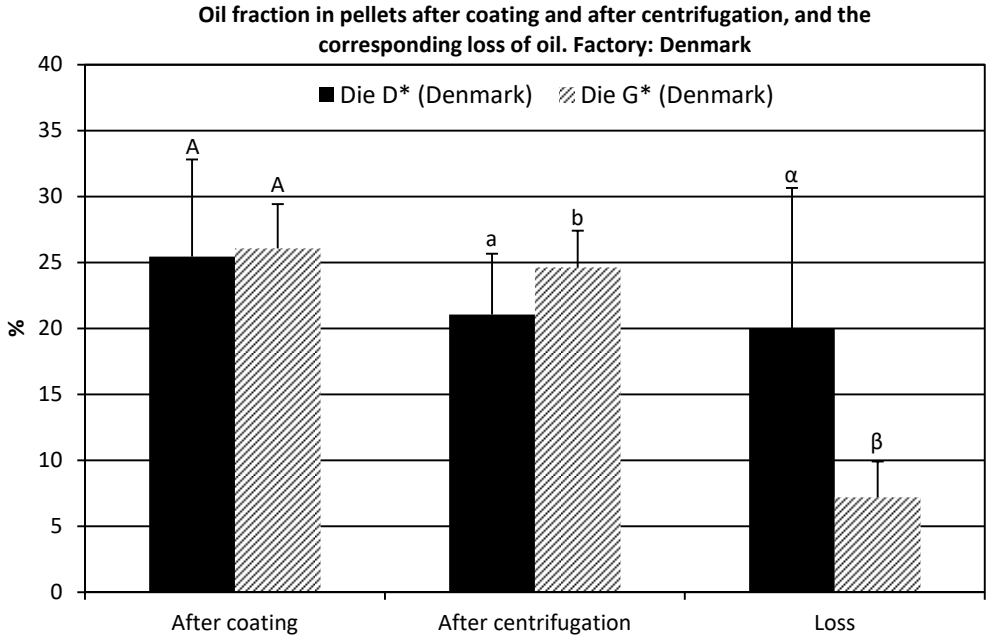

Fig. 24

Results from SPC of pellets from the Danish factory produced using Die D* and Die G*. Eight pellets from each group were treated. There is no significant difference between the two groups of pellets regarding the fraction of oil added to the pellets during coating. However, the fraction of oil after centrifugation is significantly higher for the pellets produced using Die G* than for Die D*. Also, the corresponding oil loss is significantly smaller for the pellets produced using Die G* than for Die D*.

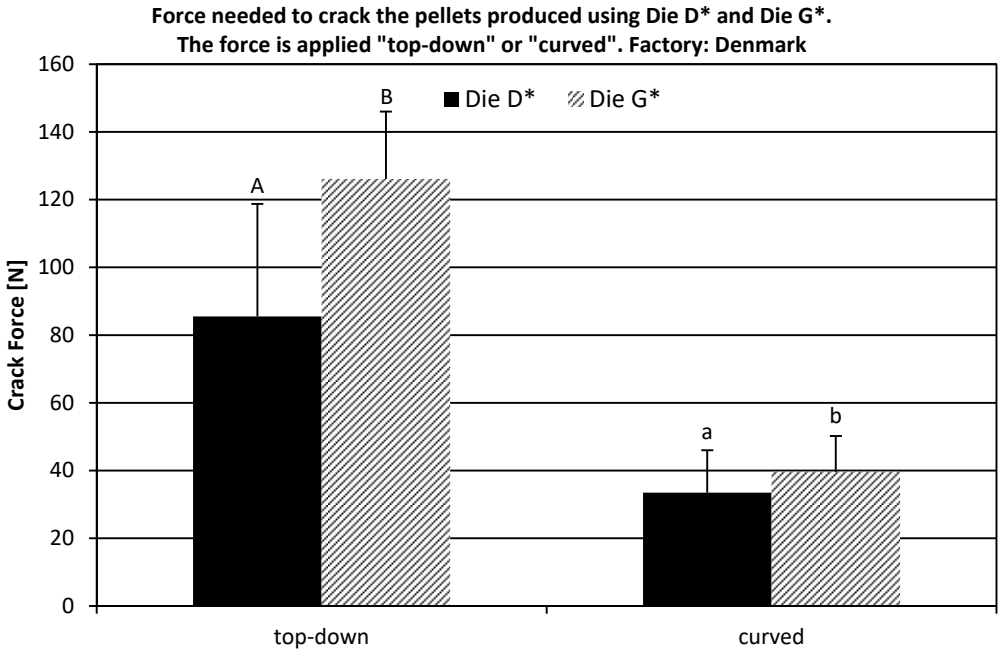

Fig. 25
The force needed to crack the pellets with the probe on a Texture Analyzer was measured for 100 pellets from each group. 50 pellets were exposed to the probe along the direction of the axial expansion (referred to as “top-down”) and 50 pellets along the direction of the radial expansion (referred to as “curved”). In both cases, the pellets produced using Die G* resisted significantly higher applied force before cracking than the pellets produced using Die D*.

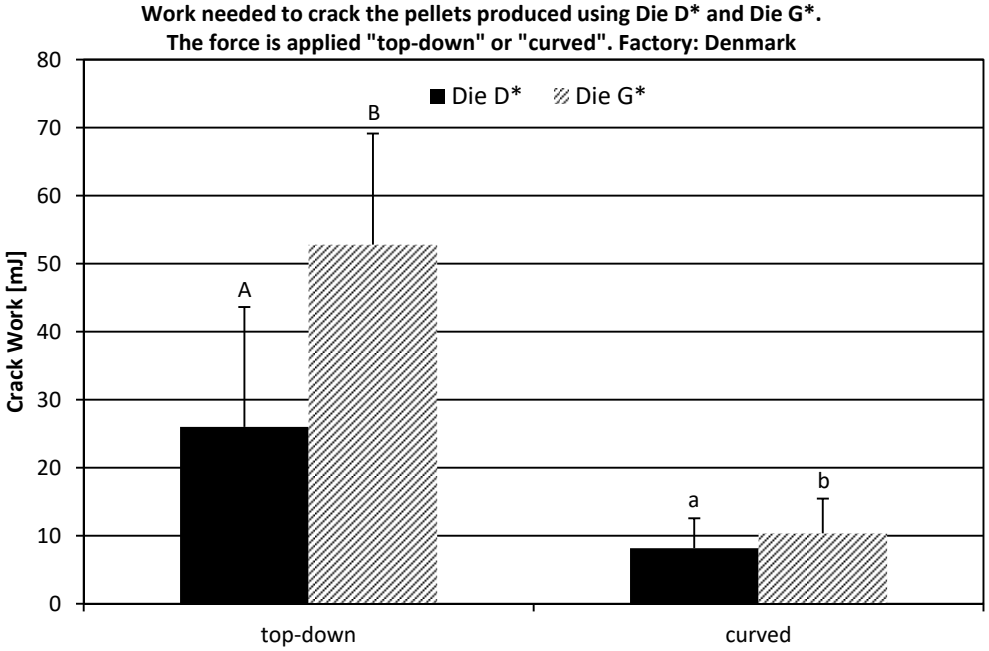

Fig. 26
The work needed to crack the pellets with the probe on a Texture Analyzer was measured for 100 pellets from each group. 50 pellets were exposed to the probe along the direction of the axial expansion (referred to as “top-down”) and 50 pellets along the direction of the radial expansion (referred to as “curved”). In both cases, the pellets produced using Die G* resisted significantly more applied work before cracking than the pellets produced using Die D*.

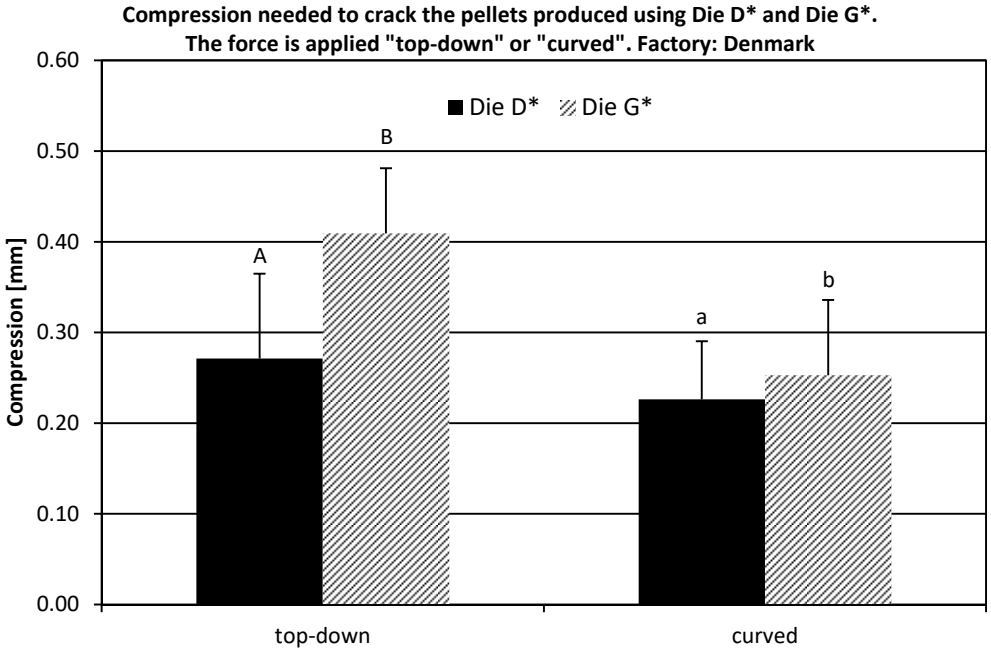

Fig. 27
The compression needed to crack the pellets with the probe on a Texture Analyzer was measured for 100 pellets from each group. 50 pellets were exposed to the probe along the direction of the axial expansion (referred to as “top-down”) and 50 pellets along the direction of the radial expansion (referred to as “curved”). In both cases, the pellets produced using Die G* resisted significantly more compression before cracking than the pellets produced using Die D*.

The force in N, work in mJ and compression in mm needed to crack the pellets produced in Denmark using Die D* and Die G* are presented in *fig. 25-27*. In all three cases, the pellets produced using Die G* resist significantly higher force, work and compression – independent of the direction of the applied stress – before cracking than the corresponding pellets produced using Die D*. This indicates that the pellets produced using the new die, Die G*, are more robust than pellets produced using traditional dies.

After the successful full scale trials in Chile and Denmark, proving the benefits by using the new die design, the process of writing the *Patent Application II* was initiated. Reference is made to this application in the confidential thesis, detailing specific wording, protected principles and defined claims.

7. Discussion

The findings from *Paper I* regarding the pores' ability to protect the additives and viewing the pellet structure as a delivery system formed the basis for *Patent Application I*, which outlined the opportunity for utilizing the pores to store probiotic bacteria and detailed a procedure to protect heat-labile additives against degradation during coating of warm pellets. The capacity of the pores appeared to be still unexploited, and the solution was to develop the next generation dies to enable full utilization of the pore potential. This work led to *Patent Application II*, which as the crowning touch addresses the PPQ challenges and links together all the research conducted during this project. Therefore, primarily the topics related to this conclusive publication will be discussed in this section. For the most part, the results were already discussed in details during their presentation, thus making this discussion section focus on the applied methods used to obtain the presented results. Are the methods reliable, what are their limitations, how to improve them, and are we allowed to draw any conclusions based on the results obtained using these methods?

In extrusion, the common understanding of the die's impact is its ability to form the extrudate (Rossen & Miller, 1973) and – by means of cutting knifes – create pellets with specific shapes (Guy, 2001). The dies have for long been associated with the outer appearance of the pellets only, whereas the overall configuration of the extruder and composition of the meal mix have been assigned to be responsible for the inner-structure of the pellets. To challenge this understanding, reliable methods to examine and evaluate the microstructure of the pellets are needed. In principle, many of the in-house test methods are good, but the sampling consistency and data handling leave a lot to be desired. Below, the methods from *chapter 5* will be discussed:

Strength & Durability

BioMar is slowly changing from Kahl to the more reliable texture analysers. However, considering the short time to analyse one pellet by TA – especially compared to the Kahl method – it would, from a statistical perspective, be praiseworthy to test more than 10 pellets. Also, I have experienced that suspiciously bad measurements are excluded, whereas suspiciously good measurements are included in the quality control. Of course, this procedure should be avoided, and it gets worse when the result is only delivered as an average. Sometimes, you can request more details, but they are not as standard included. I wonder – and fear – how ordinary decision-making takes place when you have to rely on optimized average values. Regarding the DORIS to test the pellet durability, the instrument is very popular in the feed industry and is easy to use. However, only one measurement is performed. To get as much statistics as possible, I chose to evaluate the strength of the pellets only by TA. Hereby, I managed to test 100 individual pellets per batch making it possible to build a strong data set.

Oil Leakage

The in-house oil leakage test (SF) is very standardized, fast and easy, but provides no statistics. The result is calculated as an average of the oil leakage values measured for each of the two drainable centrifuge cups containing the same amount of pellets originating from the same sample. The oil leakage is calculated as the mass of leaked oil after centrifugation relative to the initial sample mass before centrifugation. The two cups of pellets are statistically handled as independent samples, but the only reason for splitting them into two, is to balance the rotor of the centrifuge. As a consequence of the in-house oil leakage procedures, the maximum SF value obtainable is equivalent to the content of oil added during coating. Consequently, if 30% of a feed sample's mass is assigned to oil added during coating, and the SF value of said feed is 1.0%, the leakage scale is only ranging from 0% to 30%. Therefore, it would make more sense to calculate the leakage as $\frac{1.0\%}{30\%}$ = 3.3%. This is why I developed the single pellet coating: The SPC oil leakage is calculated relative to the amount of oil added during coating, and the result is not only provided as a meaningless average. In combination with the correlation to the volume and surface area of the pores measured by µCT, the SPC turned out to be the best method to provide details regarding oil leakage.

X-ray Microtomography / µCT

The initial understanding of oil leakage and how it is influenced by the geometry of the pores was based upon the results acquired by µCT of 36 pellets. Even though the results correlated very well to the SPC, I experienced the importance of standardizing every single step in the computation of the image analysis. If you are not paying attention and not following a standardized procedure, *e.g.* changing thresholds or resolution, the results are not comparable, and you will waste a lot of time and money on worthless fish feed pictures. Fortunately, a standardized procedure was developed and used for all the computations of the captured pellets.

Remarks

The development of new test methods made it possible to evaluate the impact of the die on the physical quality of extruded fish feed pellets. Also, the design of a new generation of dies to specifically facilitate a desired flow throughout the dies and an optimal pore structure in the pellets challenges the current understanding of the influence of the dies on the overall appearance of the extrudate. Without the new test methods, it would very likely have been impossible to conclude whether or not the Die G* was significantly better than Die D*. And even though the in-house test methods could be improved by simply increasing the number of pellets to be analysed, or by including raw data instead of only providing an average, the pellets are not individually evaluated, and consequently, a good and a bad pellet could neutralize each other. However, the new test methods – and especially the SPC – are more time consuming than the in-house tests, and further optimization of the new analyses are needed to make them more time-efficient.

8. Conclusion

It is not necessary to change the entire screw configuration in the extruder, or add certain raw materials or process aids to the meal mix to obtain the desired pore structure. Manipulating of the very last centimetres of the extruder has been proven to have a significant impact on the pore structure in extruded fish feed pellets. Additionally, changing to a new generation of dies improves significantly the overall physical quality of the feed. The volume and surface area of the pores are positively correlated to the amount of oil possible to add to the pellet structure during coating and the amount of oil retained in the pellet structure after centrifugation, respectively. Consequently, the optimal pore structure – from an oil leakage perspective – is characterized by many small pores rather than few big. This pore structure, resulting in significantly lowered oil leakage levels, was obtained using a new die design utilizing flow restrictions and increased shear to lower the apparent viscosity of the dough, facilitating the formation and desired surface-area to object-volume ratio of the pores.

Even though the Reynolds number for both the traditional and the new die is indicating a laminar flow, the profile of the flow throughout the new die is less parabolic and the maximum linear flow velocity is about 20% lower compared to the traditional die, indicating a more turbulent-like flow behaviour in the new die design. This promotes – along with the improved pore structure – stronger pellets because not all the pores are aligned with the flow direction in the die. The pellets produced using the new die resisted significantly higher applied force, more work and larger compression before cracking than pellets produced by the traditional die. The reduced parabolic flow profile in the new die will theoretically reduce unintended floating for smaller pellets and reduce the creation of dust related to the concave pellet bottom.

In an overall perspective, the new die design improves the physical quality of extruded fish feed pellets and allows for better utilization of the pellet pores. The die is not only responsible for the appearance of the extrudate; it is equally responsible for the creation and structure of the pore network. Therefore, it is reasonable to update or specify the previously presented extrusion definition by Rossen & Miller (1973), cf. *footnote 7*, to:

> *Food extrusion is a process in which a food material is forced to flow, under one or more of a variety of conditions of mixing, heating, and shear, through a die which is designed to form the functional shape and inner-structure of the extrudate and/or puff-dry said extrudate.*

9. Bibliography

Alltech (2017): *2017 Alltech Global Feed Survey*. 3031 Catnip Hill Road, Nicholasville, Kentucky 40356, USA.

Alvarez-Martinez, L., K.P. Kondury & J.M. Harper (1988): A General Model for Expansion of Extruded Products. *Journal of Food Science*. Vol. 53(2), pp. 609-615.

Awuah, G.B., H.S. Ramaswamy & A. Economides (2007): Thermal processing and quality: Principles and overview. *Chemical Engineering and Processing*. Vol. 46(6), pp. 584-602.

Azaza, M.S., M.N. Dhraief, M.M. Kraiem & E. Baras (2010): Influences of food particle size on growth, size heterogeneity, food intake and gastric evacuation in juvenile Nile tilapia, *Oreochromis niloticus*, L., 1758. *Aquaculture*. Vol. 309(1-4), pp. 193-202.

Bandyopadhyay, S. & R.K. Rout (2001): Aquafeed Extrudate Flow Rate and Pellet Characteristics from Low-Cost Single-Screw Extruder. *Journal of Aquatic Food Product Technology*. Vol. 10(2), pp. 3-15.

Belitz, H.-D., W. Grosch & P. Schieberle (2004): *Food Chemistry*. 3rd ed., Springer-Verlag Berlin Heidelberg, Germany. ISBN-13: 978-3-540-40818-5.

Boon, C.S., D.J. McClements, J. Weiss & E.A. Decker (2010): Factors Influencing the Chemical Stability of Carotenoids in Foods. Critical Reviews in Food Science and Nutrition. Vol. 50(6), pp. 515-532.

Brown, E.E. (1977): *World Fish Farming: Cultivation and Economics*. The AVI Publishing Company, Inc., Westport, Connecticut, USA.

Cheng, H. & A. Friis (2010): Modelling extrudate expansion in a twin-screw food extrusion cooking process through dimensional analysis methodology. *Food and Bioproducts Processing*. Vol. 88, pp. 188-194.

Chessari, C.J. & J.N. Sellahewa (2001): Effective process control. I: R. Guy (ed.) (2001): *Exstrusion cooking, Technologies and applications*. Woodhead Publishing Limited, Abington Hall, England, pp. 83-107. ISBN-13: 978-1-8-5573559-0.

Clker.com (2017): *Eppendorf Tube clip art* [online]. [Quoted July 2, 2017]. Available on: http://www.clker.com/clipart-eppendorf-tube.html

Cornel, G.E. & F.G. Whoriskey (1993): The effect of rainbow-trout (*Oncorhynchus mykiss*) cage culture on the water quality, zooplankton, benthos and sediments of Lac du Passage, Quebec. *Aquaculture*. Vol. 109(2), pp. 101-117.

Dethlefsen, M.W., N.H. Hjermitslev, S. Frosch & M.E. Nielsen (2016): Effect of storage on oxidative quality and stability of extruded astaxanthin-coated fish feed pellets. *Animal Feed Science and Technology*. Vol. 221, pp. 157-166.

Ding, Q.-B., P. Ainsworth, G. Tucker & H. Marson (2005): The effect of extrusion conditions on the physicochemical properties and sensory characteristics of rice-based expanded snacks. *Journal of Food Engineering*. Vol. 66, pp. 283-289.

Ding, Q.-B., P. Ainsworth, A. Plunkett, G. Tucker & H. Marson (2006): The effect of extrusion conditions on the functional and physical properties of wheat-based expanded snacks. *Journal of Food Engineering*. Vol. 73, pp. 142-148.

Djurhuss, J.C., K. Bock, P. Olesen, J.M. Clausen & C. Simonsen (2012): Open Access policy for public-sector research councils and foundations. *Ministry of Higher Education and Science*. Copenhagen, June 21, 2012.

Draganovic, V., A.J. Van der Goot, R. Boom & J. Jonkers (2013): Wheat gluten in extruded fish feed: effects on morphology and on physical and functional properties. *Aquaculture Nutrition*. Vol. 19(6), pp.845-859.

EU, 2017: Commission Implementing Regulation (EU) 2017/962 of 7 June 2017 suspending the authorisation of ethoxyquin as a feed additive for all animal species and categories. *Official Journal of the European Union*. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32017R0962

Frame, N.D. (1994): Operational characteristics of the co-rotating twin-screw extruder. I: N.D. Frame (ed.) (1994): *The Technology of Extrusion Cooking*. Chapman & Hall, pp. 1-51. ISBN-13: 978-0-751-40090-8.

Guy, R. (2001): Snack foods. I: R. Guy (ed.) (2001): *Exstrusion cooking, Technologies and applications*. Woodhead Publishing Limited, Abington Hall, England, pp. 161-181. ISBN-13: 978-1-8-5573559-0.

Hansen, S. (2003): *Anvendt fysik*. 3rd ed., KVL-bogladen. ISBN-13: 978-87-7432-591-8.

Hernández, A., B.G. García, M.J. Jordán & M.D. Hernández (2014): Natural antioxidants in extruded fish feed: Protection at different storage temperatures. *Animal Feed Science and Technology*. Vol. 195, pp. 112-119.

Hjermitslev, N.H. (2017): [Personal communication] Knowledge passed on during supervision and co-work throughout the present project. Niels Harthøj Hjermitslev has more than 27 years of experience within the aquaculture industry and works as senior scientist at BioMar A/S, Brande. E-mail: nhh@biomar.dk

Haubjerg, A.F. (2016): *Ensuring Technical Product Quality in the Energy Efficient Hot Air Drying of Extruded Fish Feed*. University of Southern Denmark, Philosophiae Doctor Thesis, 2016:4. ISBN-13: 978-87-93192-90-4

Horvat, M., M.A. Emin, B. Hochstein, N. Willenbacher & H.P. Schuchmann (2013): A multiple-step slit die rheometer for rheological characterization of extruded starch melts. *Journal of Food Engineering*. Vol. 116(2), pp. 398-403.

Jokumsen, A. & L.M. Svendsen (2010): *Farming of Freshwater Rainbow Trout in Denmark*. Report no. 219-2010. DTU Aqua, Charlottenlund, National Institute of Aquatic Resources, Technical University of Denmark.

Kazemzadeh, M. (2011): Introduction to Extrusion Technology. I: A. Maskan & M. Altan (eds.) (2011): *Advances in Food Extrusion Technology*. CRC Press, Taylor & Francis Group 6000 Broken Sound Parkway NW, Suite 300, pp. 1-21. ISBN-13: 978-1-4398-1521-2.

Kokini, J.L., C.N. Chang & L.S. Lai (1992): The Role of Rheological Properties on Extrudate Expansion. I: J.L. Kokini, C.-T. Ho & M.V. Karwe (eds.) (1992): *Food Extrusion Science and Technology*. Marcel Dekker, Inc., 270 Madison Avenua, New York, New York 10016, USA, pp. 631-652. ISBN-13: 978-0-824785-42-0.

Kraugerud, O.F., H.Y. Jørgensen & B. Svihus (2011): Physical properties of extruded fish feed with inclusion of different plant (legumes, oilseeds, or cereals) meals. *Animal Feed Science and Technology*. Vol. 163, pp. 244-254.

Lam, C.D. & R.A. Flores (2003): Effect of Particle Size and Moisture Content on Viscosity of Fish Feed. *Cereal Chemistry*. Vol. 80(1), pp. 20-24.

Launay B. & J.M. Lisch (1983): Twin-screw Extrusion Cooking of Starches: Flow Behaviour of Starch Pastes, Expansion and Mechanical Properties of Extrudates. *Journal of Food Engineering*. Vol. 2, pp. 259-280.

Liu, Q. (2005): Understanding Starches and Their Role in Foods. I: S.W. Cui (ed.) (2005): *Food Carbohydrates, Chemistry, Physical Properties, and Applications*. CRC Press, Taylor & Francis Group 6000 Broken Sound Parkway NW, Suite 300, pp. 309-356. ISBN-13: 978-0-8493-1574-9.

Ma, H., Z. Pan, B. Li, B.B. Atungulu, D.A. Olson, M.M. Wall & T.H. McHugh (2012): Properties of extruded expandable breadfruit products. *LWT – Food Science and Technology*. Vol. 46, pp. 326-334.

MetroXpress (2013): Advertisement: *"Forstå det. Gør noget ved det."*. Thursday, February 21, 2013, MetroXpress 12(37), p. 8. Copenhagen.

Miller, J.C. (1963): Swelling Behavior in Extrusion. *Polymer Engineering & Science*. Vol. 3(2), pp. 134-137.

Miller, R.C. (1994): Breakfast and cereal extrusion technology. I: N.D. Frame (ed.) (1994): *The Technology of Extrusion Cooking*. Chapman & Hall, pp. 73-109. ISBN-13: 978-0-751-40090-8.

Moraru, C.I. & J.L. Kokini (2003): Nucleation and Expansion During Extrusion and Microwave Heating of Cereal Foods. *Comprehensive Reviews in Food Science and Food Safety*. Vol. 2, pp. 147-165.

Newton, I. (1675): Letter from Sir Isaac Newton to Robert Hooke. Cambridge, February 5, 1675. http://digitallibrary.hsp.org/index.php/Detail/Object/Show/object_id/9285

Riaz, M.N. (2000): Introduction to Extruders and Their Principles. I: M.N. Riaz (ed.) (2000): *Extruders in Food Applications*. Technomic Publishing Company, Inc., Lancaster, Pennsylvania 17604, USA, pp. 1-23. ISBN-13: 978-1-5-6676779-8.

Risum, J. & A. Friis (2009): *Food Process Engineering – An Introduction*. 3rd ed., DTU Food. ISBN-13: 978-87-92158-27-7.

Robin, F., C. Dubois, N. Pineau, E. Labat, C. Théoduloz & D. Curti (2012): Process, structure and texture of extruded whole wheat. *Journal of Cereal Science*. Vol. 56, pp. 358-366.

Rossen, J.L. & R.C. Miller (1973): Food Extrusion. *Food Technology*. Vol. 27(8), pp. 46-53.

Singh, M.N., K.S.Y. Hemant, M. Ram & H.G. Shivakumar (2010): Microencapsulation: A promising technique for controlled drug delivery. *Research in Pharmaceutical Sciences*. Vol. 5(2), pp. 65-77.

Sokhey, A.S., Y. Ali & M.A. Hanna (1997): Effects of Die Dimensions on Extruder Performance. *Journal of Food Engineering*. Vol. 31, pp. 251-261.

Sørensen, M. (2012): A review of the effects of ingredient composition and processing conditions on the physical qualities of extruded high-energy fish feed as measured by prevailing methods. *Aquaculture Nutrition*. Vol. 18(3), pp. 233-248.

Sørensen, M., G. Nguyen, T. Storebakken & M. Øverland (2010): Starch source, screw configuration and injection of steam into the barrel affect the physical quality of extruded fish feed. *Aquaculture Research*. Vol. 41(3), pp. 419-432.

Tacon, A.G.J. & I.P. Forster (2003): Aquafeeds and the environment: policy implications. *Aquaculture*. Vol. 226(1-4), pp. 181-189.

Tacon, A.G.J. & M. Metian (2008): Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture*. Vol. 285(1-4), pp.146-158.

The World Bank (2013): *Fish to 2030. Prospect for Fisheries and Aquaculture*. World Bank Report Number 83177-GLB. The World Bank, 1818 H Street NW, Washington DC 20433, USA.

Thomas, M., T. van Vliet & A.F.B. van der Poel (1998): Physical quality of pelleted animal feed 3. Contribution of feedstuff components. *Animal Feed Science and Technology*. Vol. 70(1-2), pp. 59-78.

Torrissen, O.J. (1985). Pigmentation of salmonids: factors affecting carotenoid deposition in rainbow trout (*Salmo gairdneri*). *Aquaculture*. Vol. 46(2), pp. 133-142.

Wachowski, L. & L. Wachowski (1999): *The Matrix*. Warner Bros., Village Roadshow Pictures, Groucho II Film Partnership, and Silver Pictures. USA.

Wankowski, J.W.J. & J.E. Thorpe (1979): The role of food particle size in the growth of juvenile Atlantic salmon (*Salmo salar* L.). *Journal of Fish Biology*. Vol. 14(4), pp. 351-370.

Wolska, J. (2014): Towards a better understanding and control of factors affecting the physical quality of fish feed. Department of Biotechnology, Lund University. PhD thesis. ISBN-13: 978-91-7422-374-3.

Williams, M.A. (2000): Interrupted-Flight Expanders-Extruders. I: M.N. Riaz (ed.) (2000): *Extruders in Food Applications*. Technomic Publishing Company, Inc., Lancaster, Pennsylvania 17604, USA, pp. 63-79. ISBN-13: 978-1-5-6676779-8.

Aas, T.S., M. Oehme, M. Sørensen, G. He, I. Lygren & T. Åsgård (2011): Analysis of pellet degradation of extruded high energy fish feeds with different physical qualities in a pneumatic feeding system. *Aquaculture Engineering*. Vol. 44(1), pp. 25-34.

Animal Feed Science and Technology 221 (2016) 157–166

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

Effect of storage on oxidative quality and stability of extruded astaxanthin-coated fish feed pellets

Markus Wied Dethlefsen [a,c], Niels Harthøj Hjermitslev [a], Stina Frosch [b], Michael Engelbrecht Nielsen [c,d,*]

[a] Biomar A/S, R&D Process Technology, Mylius Erichsensvej 35, 7330 Brande, Denmark
[b] Division of Industrial Food Research, Food Process Engineering Research Group, National Food Institute, Technical University of Denmark, Søltofts Plads, 2800 Kgs. Lyngby, Denmark
[c] Biological Quality Research Group, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, 2860 Søborg, Denmark
[d] Fast-Q, R&D, Sagasvej 2A, 1861 Frederiksberg, Denmark

ARTICLE INFO

Article history:
Received 5 October 2015
Received in revised form 3 August 2016
Accepted 5 August 2016

Keywords:
Fish feed
Extrusion
Coating
Astaxanthin
Photo-oxidation
Color degradation

ABSTRACT

This study examined the stability of extruded and astaxanthin-coated fish feed pellets during storage in a light box at 28 °C and 620 lx. Seven groups of fish feed pellets were vacuum coated with fish oil that contained levels of astaxanthin ranging from 0 to 100 ppm. To equalize differences in the conditions for the fish feed pellets inside the light box, the samples were systematically circled during the experimental storage period of 183 days. The degradation of astaxanthin was monitored using multi-spectral images, captured 28 times in the course of the storage period. Additionally, samples were collected at storage day 8, 15, 22, 92 and 183 for chemical determination of the astaxanthin concentration. The degradation of astaxanthin was shown to primarily be affected by light and limited to occur at the surface of the fish feed pellets, whereas the astaxanthin embedded in the core of the pellets was comparatively protected against degradation. Furthermore, the initial concentrations of astaxanthin influenced the degradation *per se*, signifying self-protective properties of astaxanthin.

1. Introduction

Flesh pigmentation, along with freshness, is well-known to be the major quality parameter when evaluating the properties of salmonids for human consumption (Torrissen and Naevdal, 1988; Torrissen and Christiansen, 1995). Astaxanthin is the primary pigment accounting for the natural red colour of wild salmon, lobster, crab and shrimp. Commercially produced astaxanthin is the carotenoid pigment used in fish feed for the salmonid aquaculture industry (Sigurgisladottir et al., 1994). When farmed, the fish are supplied with pigment through the diet in concentrations ensuring a desired colour profile of the flesh at slaughtering. The right degree of flesh pigmentation is of great economic importance for the individual farmer (Torrissen, 1985), wherefore the requirements for the feed producers regarding pigmentation efficiency and predictability are strict. Astaxanthin is exposed to a range of harsh conditions during processing and storage of feed, including heat, light

* Corresponding author at: Fast-Q, R&D, Sagasvej 2A, 1861 Frederiksberg, Denmark.
E-mail addresses: mwd@biomar.dk (M.W. Dethlefsen), nhh@biomar.dk (N.H. Hjermitslev), sfro@food.dtu.dk (S. Frosch), mice@food.dtu.dk, men@fast-q.dk (M.E. Nielsen).

http://dx.doi.org/10.1016/j.anifeedsci.2016.08.007
0377-8401/

Table 1
Astaxanthin concentration in coating oil and grouping of 35 samples.

Dilution	Fraction of K7	Group name	Sample ID
0 ppm	0	K1	1–5
5th dilution	1/32	K2	6–10
4th dilution	1/16	K3	11–15
3rd dilution	1/8	K4	16–20
2nd dilution	1/4	K5	21–25
1st dilution	1/2	K6	26–30
100 ppm	1	K7	31–35

and oxygen, which all have influence on the final concentration of astaxanthin in the fish feed (Marleen, 2007; Armenta and Guerrero-Legarreta, 2009; Franco-Zavaleta et al., 2010; Anarjan and Tan, 2013). The deposition rate of astaxanthin in the flesh is known to be less effective as the astaxanthin concentration in the feed is increased; yet, the overall deposition is found to be larger for feed with high concentrations of astaxanthin (Choubert and Storebakken, 1989; Hencken and Estermann, 1991). Previously, astaxanthin was added directly to the meal mix prior to extrusion of the fish feed. As a result of the high extrusion temperature, nearly 50% of the astaxanthin was lost in this step of the process alone (Hencken and Estermann, 1991). Nevertheless, absence of antioxidants during extrusion can result in formation of free radicals (Schaich, 2002). Even though the harm of the radicals is limited in the extruder, they can initialize the oxidation of lipids and valuable nutrients, which are added in the subsequent coating, and thereby reducing the oxidative stability of the feed. Reduced oxidative stability of fish feed is known to affect the growth and health of the fish as well as their nutritional quality (Hernández et al., 2014).

Historically, astaxanthin is a very expensive feed additive, and considering the negligible mass fraction posed in the recipes for salmonid feed, it nevertheless made up for 10–15% of the total feed costs in the early 1990s (Johnson and An, 1991). Even though price of astaxanthin has decreased considerable within the recent years, determination of astaxanthin losses during processing, handling and storage of the feed are of outmost interest for prediction of the ability of fish feed to colour the flesh of fish.

Today, the general practice of astaxanthin inclusion is to dispense astaxanthin with fish oil and subsequent coat the fish feed pellets with the mixture. This process reduces heat-related losses during extrusion. The method of coating for the incorporation of pigment into the feed places specific demands to the pellets' ability to protect and preserve astaxanthin. The aim of this study is to investigate the stability of extruded and astaxanthin-coated fish feed pellets during storage, and to evaluate the self-protective properties of the pellets. Inspired by Boon et al. (2010), the pellet structure can, from the perspective of the components in the coating fraction and the fish as the recipient, be seen as a delivery system for the valuable and fragile feed ingredients, including astaxanthin. Recently, Anarjan and Tan (2013) examined the effects of temperature, atmosphere and light on the stability of astaxanthin nano-dispersions during storage. They prepared the samples as emulsions and stored them in glass vials. Regardless of the easy reproducibility, this setup is less representative to actual conditions present during storage of astaxanthin-containing food and feed products. Thus, the surrounding matrix and its possible interference or properties as a delivery system are not considered. Therefore, evaluation of astaxanthin stability in feed pellets should be investigated in the relevant matrix in contrast to the proposed glass vial approached by Anarjan and Tan (2013).

2. Material and methods

2.1. Materials, apparatus and observations

2.1.1. Materials

Seven groups of fish feed pellets, each containing five samples, were coated with different concentrations of astaxanthin. The in total 35 samples were systematically arranged in a storage box with controlled continues light for 183 days. Each sample contained 40 g of extruded pellets, originating from the same batch of fish feed. The fish feed pellets were produced using a commercial twin-screw extruder (Clextral BC45, Clextral, France) under normal commercial conditions set to a pellet size of 4.5 mm. Despite natural present antioxidants in the meal mix, 100 ppm of ethoxyquin (Impexquin 6S, Art.: 05210000, Impextraco, Belgium) was added to the recipe. After extrusion, the group of pellets were vacuum coated (cf. section 2.1.2. *Coating Procedure*) with fish oil containing different concentrations of synthetic cold water dispersible astaxanthin (Lucantin® Pink CWD, Art.: 51027961, BASF, Germany). The highest concentration of astaxanthin was 100 ppm and was used for the group named K7. The coatings used for group K2-K6 were produced by dilution of K7 with fish oil, so the concentration of astaxanthin each time was diluted to the half of the previous concentration. Group K1 was coated with pure fish oil and is defined to contain 0 ppm of astaxanthin (Table 1). Astaxanthin is commonly measured in ppm, and relates to mass, so ppm corresponds to milligram per kilogram. After production, the pellets were stored for two months in vacuum-packed plastic bags at 2 °C in a dark environment to minimize the oxidation process prior to the illuminated 183 days in the storage box. The two months of pre-storage were necessary to solve logistical and executional challenges across academia and industry.

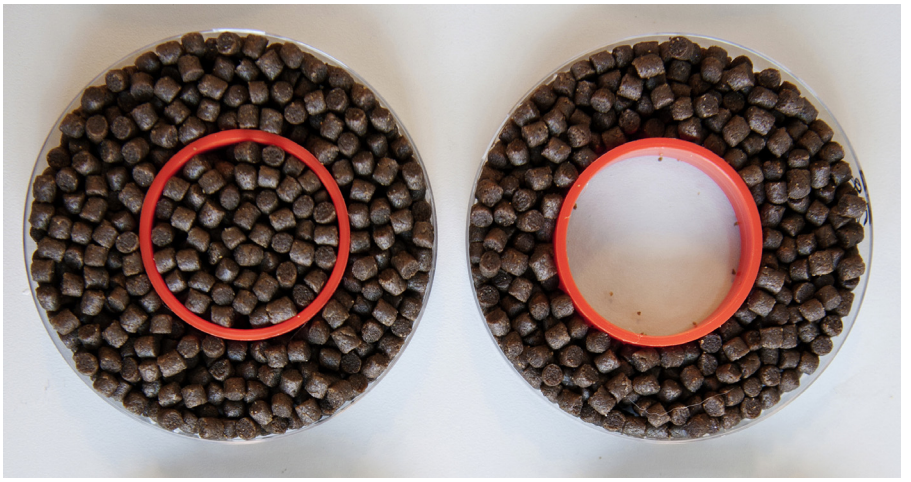

Fig. 1. Zoning of Petri dishes using red plastic rings to separate fish feed pellets for chemical analysis and multi-spectral imaging. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.1.2. Coating procedure

To produce the different coatings, the astaxanthin was dissolved in water and then diluted with fish oil until the concentration used for K7 was obtained. Performing the dilution of K7 to produce the coatings used for K2-K6 resulted in a minor reduction of the water content in the coatings. The coating was conducted under vacuum in a specially designed vacuum coater for experimental usage (100 mbar, 55 °C, 90 s vacuum-release) to introduce as much coating into the pellets as possible. Pellets were placed in an airtight chamber equipped with a valve and a hose. First, the pellet filled chamber was vacuumed and then, the valve was opened allowing the coating to be sucked in by the vacuum while the chamber was continuously being rotated.

2.1.3. Apparatus

2.1.3.1. Design of sample unit. Zoned Petri dishes of plastic (Nunc™ Petri Dishes, NUNC, Denmark) with a diameter of 9 cm served as containers for each of the 35 samples. The Petri dishes were filled with fish feed pellets just above the lip to let the lid rest on them. A red plastic ring was introduced to divide the dishes into two zones: An inner and an outer area, as shown in Fig. 1. This separation of the pellets within the sample, and placement and property of the ring, served various purposes: The pellets contained in the inner zone were reserved for chemical analysis whereas pellets in the outer zone were used for the multi-spectral images. The design enabled sampling of subsamples for chemical analyses without affecting appearance of surrounding pellets. Thus, the remaining pellets in the outer zone would continue unaffected through the storage and continuously contribute to the multi-spectral images. The inner zone was never included in the processing of the multi-spectral images. This zone contained approximately 10 g of fish feed pellets, which were used only for the chemical analysis.

2.1.3.2. Storage light box. During the storage period, the samples were arranged in a closed box to control the light and the position of the dishes; inner dimensions (HxWxD): 47 cm × 65 cm × 65 cm. The lid of the box was designed to shade for the outside coming light, and had two fluorescent lamps emitting natural daylight (Osram Biolux®, L18 W/965) and a light intensity/thermometer logging device mounted on the inside (HOBO Pendent®, Temperature/Light Data Logger 64 K, UA-002-64). At the bottom of the box, a stamped out tray with 36 holes was mounted to keep the 35 Petri dishes systematically arranged. The last of the fields was used for a second HOBO-Logger identical to the one placed on the lid; both were logging the lux and the temperature every 15 min. The installation is shown in Fig. 2.

2.1.3.3. VideometerLab. Multi-spectral images of the samples were captured using a VideometerLab (Videometer A/S, Scorpion SCOR-20SOM, Denmark). The camera has 19 spectral bands (395, 435, 450, 470, 505, 525, 570, 590, 630, 645, 660, 700, 850, 870, 890, 910, 940, 950 and 970 nm), and a resolution of 960 × 1280 pixels. All images were saved in the standard raw VideometerLab format as *.hips*-files. The VideometerLab was calibrated weekly and was turned on one hour before sampling to ensure colour temperature uniformity when pictures were captured.

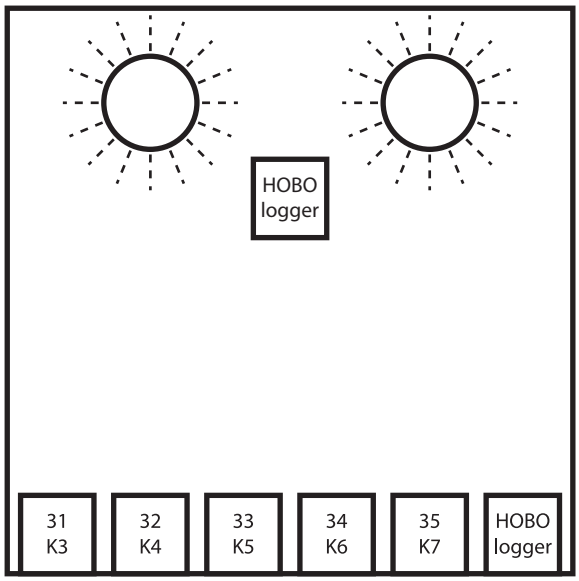

Fig. 2. Vertical cross section of the storage light box showing the front row of samples, the logging devices and the fluorescent tubes.

2.2. Analytical procedure

2.2.1. Start of sampling

During the storage experiment, the light box provided controlled conditions regarding temperature and light intensity, 28 °C ± 0.46% and 620 lx ± 34%, respectively (n = 17,568). To ensure stability of the samples in relation to water evaporation, the first sampling occurred after six days of acclimation.

2.2.2. Sampling technique

All 35 samples were measured 28 times during the 183 days of the experiment. At storage day 8, 15, 22, 92, and 183, the content of the inner zone was sampled from each group to determine the astaxanthin concentration by chemical analysis (cf. section 2.2.4. *Chemical Analysis for Astaxanthin*). For astaxanthin determinations, pellets were removed from the inner zone, vacuum-packed and stored in a dark environment at 2 °C for further analysis.

At each sampling point, all Petri dishes were weight (Vibra Shinko Denshi, Max/d 5000/0.02 g, Japan) and degree of stickiness of the pellets was quantified (cf. section 2.2.5.2 *Stickiness of Pellets*). To minimize procedure deviations, the same person performed the 28 inspections and samplings. To minimize potential unequal light intensity inside the storage light box, all samples were moved a slot once a week, including the bottom-placed HOBO-Logger. The initial positioning and the weekly rotation pattern are shown in Fig. 3.

2.2.3. Data processing of multi-Spectral images

2.2.3.1. Data processing of whole pellet images. Data processing of multi-spectral images was carried out using Matlab 7.13 (The Mathworks Inc., Natick, MA, USA). A script masking out the region of interest (ROI) was established for automation of the procedure. First, the script excludes the inner zone taking advantage of the fully reflection in the tenth band (645 nm) of the red ring. Then, all the shadow areas with unequal reflection along with the edge and outer area of the Petri dish were removed, leaving approximately 50% of the image for further extraction as shown in Fig. 4. The mean spectrum of the remaining pixels was then exported for the 19 individual layers and saved along with wavelengths and sample date. For representation, the result of each group is calculated as the average of five measurements.

2.2.3.2. Data processing of ground pellet images. The multi-spectral images recorded of the whole pellets during the storage period only contain information about the surface of the upper layer pellets. To evaluate the overall average colour including the core of the pellets, approximately 100 g of the samples from each group was ground for 60 s at the end of the storage period (Mini Chopper from OBH, type 6720, 230 V, 150 W, 4500–5000 RPM) and multi-spectral images were captured according to the previously described method.

2.2.4. Chemical analysis for astaxanthin

The HPLC analysis for astaxanthin determination was performed in duplicates at the National Food Institute, Division of Industrial Food Research at the Technical University of Denmark (Lyngby, Denmark) according to the procedure described by Ljungqvist et al. (2014).

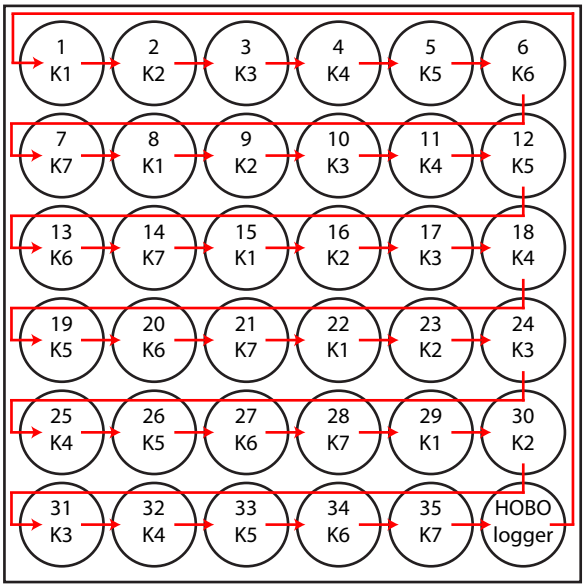

Fig. 3. Initial arrangement of samples and HOBO-Logger in the storage light box and the rotation direction.

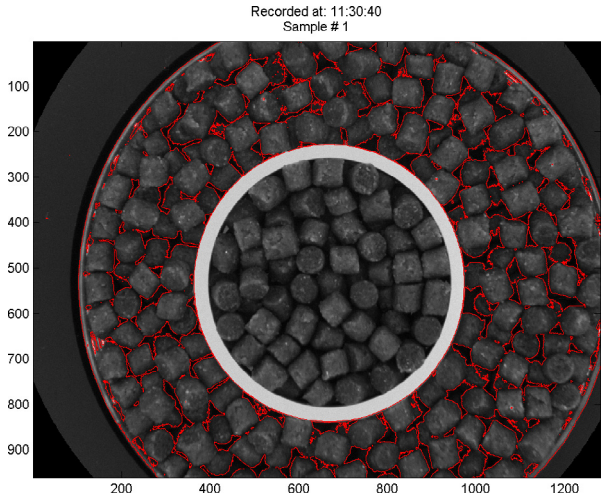

Fig. 4. Example of processed multi-spectral image marked with metadata and ROIs.

2.2.5. Physical observations

2.2.5.1. Differences in pellet colour. In addition to the data from the multi-spectral images, visually conspicuous observations were registered during sample handling, including: 1) Comparison of redness of fish feed pellets from the top and bottom layer pellets in the Petri dishes. 2) Changes in redness of fish feed pellets during vacuum packaging as the coating from the pellet core was partly sucked out. For both registrations, the evaluation is based on comparative ranking across the seven groups of samples with five levels of redness, going from not red, slightly red, red, very red, intense red.

2.2.5.2. Stickiness of pellets. The stickiness of the fish feed pellets was noted as a physical parameter. The degree of stickiness is difficult to quantify only relying on the sampler's subjective evaluation; therefore, the quantification is the result of whether or not the top layer pellets were sticking to the lid of the Petri dishes. All Petri dishes were filled so the lid was underpinned by pellets and not the lip of the Petri dish. Furthermore, to minimize differences between the samples, the stickiness index was worked out as the total number of sticky pellets within each group rather on individual sample basis.

2.2.5.3. Statistical analysis. Data are presented as mean value ± SD (standard deviation) or mean ± SD relative to mean [%] except where stated otherwise. The software used for statistical analysis and scientific graph generating is Matlab 7.13 (The Mathworks Inc., Natick, MA, USA) and GraphPad Prism 6.0 (GraphPad Software Inc. San Diego, USA). Student's *t*-test, ANOVA

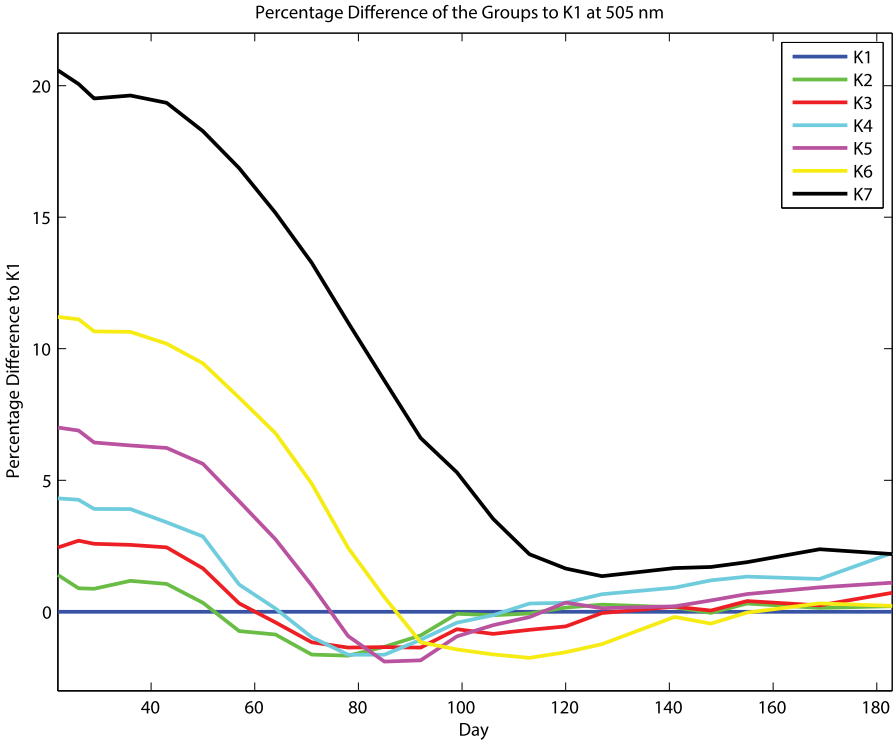

Fig. 5. The decolourization of whole pellets visualized as the colour difference to the reference group (K1) during the storage period. The time curves follow the initial level of added astaxanthin until the set in of the stationary phase.

and Friedman's test (analysis of variance) test were used for the statistical analysis. Differences were considered significant if $P < 0.05$.

3. Results

Colour differences across groups of astaxanthin-coated fish feed pellets were calculated relative to K1 at 505 nm (Fig. 5). The early colour development of group K2-K7 shows an exponential path, followed by a short steady period (lag phase) and a linear decrease (decolourization phase). At the end of the storage period, the differences between the groups were levelled out close to the baseline constituted by K1 (stationary phase). The colour differences seen in Fig. 5 at start and end correspond to Fig. 6A and B, respectively. For the whole pellets, the colour differences for K2 and K4-K7 at start are significantly different from each other, and K3 is significantly different from K5-K7. The same is not the case for the whole pellets at the end of the storage period, where the appearance of the groups essentially is insignificantly different from each other. However, the initial colour differences seen on the surface of the pellets across the samples are afresh unveiled at the end when the pellets are ground (Fig. 6C). The ground pellets at the end show the same exponential graduation as the whole pellets at start. Again, K4-K7 is significantly different from each other and K2-K3. As the appearance and range in concentration of astaxanthin changes over time (Fig. 6D–F), the percentage differences to K1 should only be compared within each subplot and not across Fig. 6A–C.

The multi-spectral images only include the surface of the upper-layer pellets whereas the pellets in the bottom of the Petri dishes are shadowed from the imaging and the light from the fluorescent tubes. By manual comparison of the visual appearance of the pellets from the upper-layer subjectively with the bottom-layer, it was clear that the decolourization of the shadowed pellets was slowed down. At the end of the storage period, the sampler was unable to distinguish the groups of pellets when studying them from above. However, they could easily be ranked after the initial astaxanthin concentration by looking at the pellets in the bottom of the Petri dishes. Another colour related observation was made in connection with the vacuum packaging of the inner zone pellets: Before the vacuum packaging, the colour of the pellets varied with their former layer position in the Petri dishes, and after packaging, when the coating from the inside of the pellets was sucked out during the vacuum, the upper layer pellets turned into the same appearance as the pellets from the bottom layer.

For each group of samples, the total number of sticky pellets is summarized in Table 2. The onset of stickiness is deferred with increased concentration of astaxanthin. The same pattern of deferment regarding the minimum relative weight was seen in Fig. 7, showing the changes in weight relative to K2 during the storage. The results show that the changes in weight are displaced after the initial concentration of astaxanthin, and higher concentrations result in larger relative loss of weight.

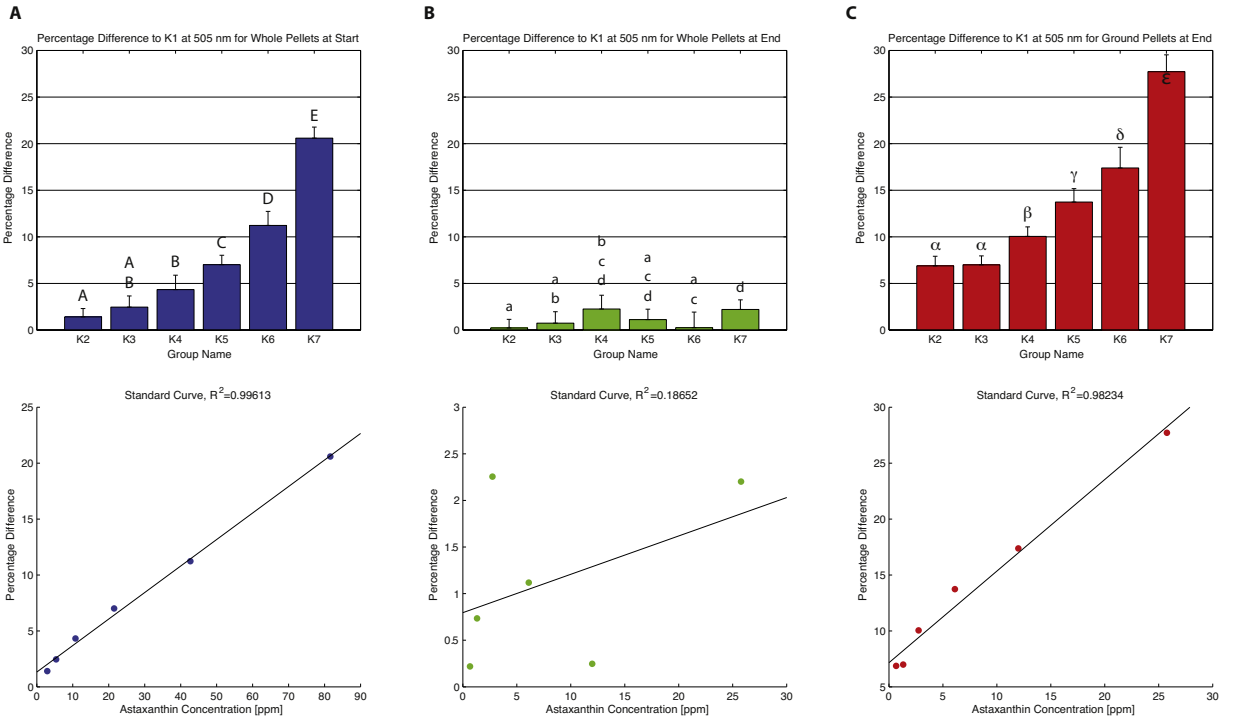

Fig. 6. A–C. The colour difference to the reference group (K1) for whole pellets at start and end, and ground pellets at end, respectively. The stationarity and level for whole pellets at end (B) indicate a uniform surface colour among the groups, and the exponential trend for whole pellets at start (A) and ground pellets at end (C) indicates that the decolourization is prevented in the core of the pellets and primary takes place on the surface. Letter explanation:.
Subplot A: A: K2-K3 is significantly different from all other groups. B: K3 is significantly different from K5-K7, and K4 is additionally significantly different from K2. C, D, E: K5-K7 is significantly different from all other groups.
Subplot B: a: K2 and K6 are significantly different from K7. b: K3 is significantly different from K7. c: K4 is significantly different from K2. d: K7 is significantly different from K2, K3 and K6. K5 is not significantly different from any other groups.
Subplot C: α: K2 and K3 are significantly different from all other groups. β, γ, δ, ε: K4-K7 is significantly different from all other groups.

Table 2
Number of sticky pellets in each group for different sampling days.

Day	K1	K2	K3	K4	K5	K6	K7
50	5						
57	27	1	5				
64	6	9	3	1			
71	10	4	7	11			
78	3	3	7		1		
85	3	2	1	2	4	3	
92	2	2	1	1	2	1	
99	3	7	3	5	3	2	
106	4	1		1	1	3	2
113	1	2	2	7	2	4	3
120	5	7	4	5	9		1
127		4	3	12	4	3	
141	2		3	9	17	9	3
148	2	4	1	6	10	9	
155	2	5	4	1	8	13	6
169		4		9	14	7	12
183	2	4	3	13	10	5	5

The results presented in Fig. 8 originate from the HPLC analyses and show that lower initial concentrations of astaxanthin significantly decreases the relative levels of astaxanthin left in the pellets at the end of the storage period in comparison to higher initial concentrations.

4. Discussion

The acclimation period equalized the initial differences in water content across the groups of fish feed pellets coated with astaxanthin-added fish oil. Introducing the acclimation period and delaying the start of monitoring the storage experiment

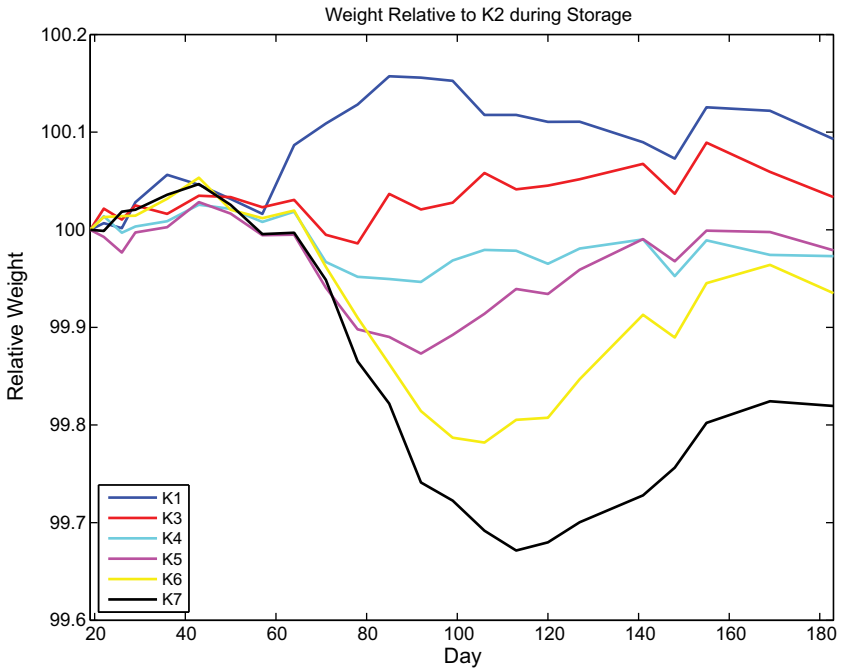

Fig. 7. Changes in weight relative to K2 during storage. The extrema are displaced after initial astaxanthin concentration, and the displacement follows the same pattern as the delay in stickiness seen in Table 2. K2 is chosen as reference as no water was added in the coating of K1.

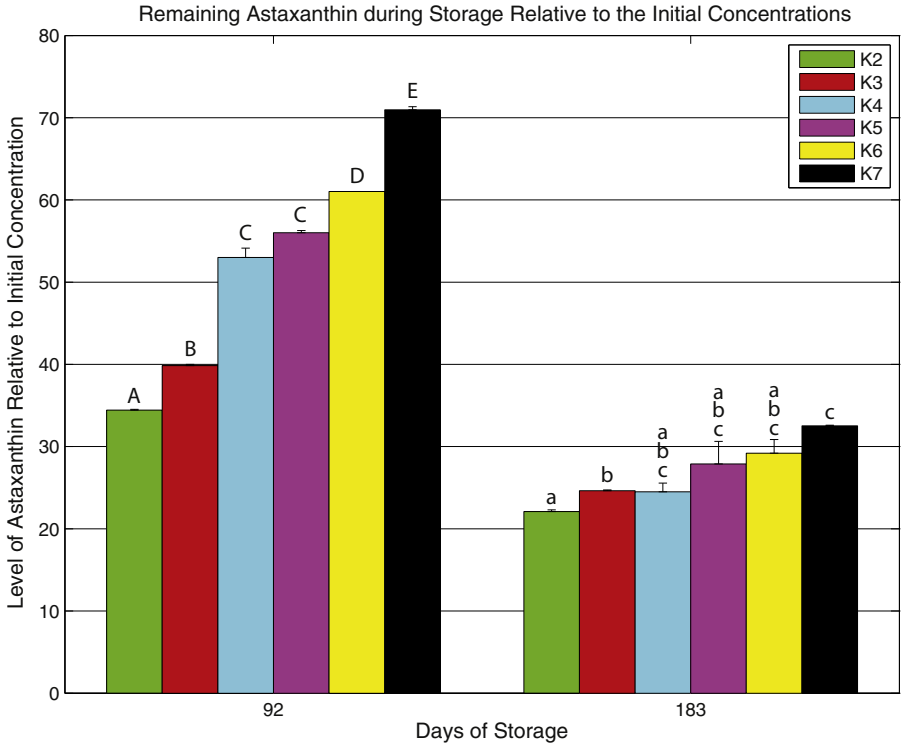

Fig. 8. The level of remaining astaxanthin relative to initial concentration was determined by chemical analysis at storage day 92 and 183. The levelling indicates that astaxanthin has self-protective properties and that higher initial concentrations provide better protection against astaxanthin degradation. A, B, D, E: K2, K3, K6 and K7 are significantly different from all other groups. C: K4 and K5 are significantly different from all other groups. a: K2 is significantly different from K3 and K7. b: K3 is significantly different from K2 and K7. c: K7 is significantly different from K2 and K3. K4-K6 is not significantly different from any other groups.

until the sixth sampling day involve lower initial measured concentrations of astaxanthin as its degradation proceeds during the acclimation as well. However, the delayed start affects no conclusions and ensures directly comparable groups at the different times and under similar sampling conditions in the rest of the storage experiment.

Based on the mean spectra for the multi-spectral surface images, it was found that the absorbance peak of the added astaxanthin was most pronounced for the fifth band in the images, corresponding to 505 nm. This is well in accordance with Ljungqvist (2012). From previous studies, it is well-established that astaxanthin is unstable, gets decolourized and is sensitive to oxidative processes, especially caused by light and oxygen (Armenta and Guerrero-Legarreta, 2009; Franco-Zavaleta et al., 2010; Anarjan and Tan, 2013). This decolourization is found to be very pronounced and all groups follow the same pattern, constituted by a lag phase, a decolourization phase, and a stationary phase, respectively. The lag phase indicates the presence of antioxidants in the fish feed pellets, which partly protect the astaxanthin from degradation. After the depletion of the antioxidants, the degradation of astaxanthin accelerates, and at the end of the storage period, the individual differences of remaining astaxanthin on the surface of the pellet samples, are very small. The same pattern of oxidation, in the presence of antioxidants, has been reported by Kitts and Tomiuk (2013).

The differences in the initial astaxanthin concentration across the groups (Fig. 6A) follow the expected pattern of a serial dilution. The reason for the deviation from the relative concentrations presented in Table 1 can be explained by the standard deviations and, more likely, as a result of already on-going degradation of astaxanthin as the initial measures in reality correspond to the sixth sampling day. Even though the groups of whole and ground pellets, represented by Fig. 6B and C, respectively, are obtained at the end of the storage period, the appearance of the pellets is very different: The whole pellets show the uniformity from the stationary phase (Fig. 5), and the colour differences among the groups of ground pellets demonstrate the same exponential trend as seen for the whole pellets at start. This suggests that astaxanthin is protected in the core of the pellets because of the absence of light.

The resistance to degradation of astaxanthin in the absence of light is consistent with the two subjective colour related observations noted during sampling. The relative differences in redness of the groups were easily visible when evaluating the shadowed pellets in the bottom of the Petri dishes and the appearance of the ground pellets at the end of the storage period. This indicates first that the decolourization mainly is induced by the light, and secondly, it supports the extracted data presented in Fig. 6C showing that the surface of the pellets protects the astaxanthin situated in the core. Ljungqvist et al. (2014) assumed that the total amount of astaxanthin in the pellets right after coating is linearly related to the initial surface reflection of the pellets. Now, knowing that a considerable part of the coating is situated and protected in the core of the pellets emphasizes that the assumption applied in Ljungqvist et al. (2014) is only valid for initial determination of the coating concentration.

As introductorily discussed, multi-spectral images can be used to evaluate the samples across the groups at the different times and under similar sampling conditions. This implies that the scale of percentage differences to K1 in Fig. 6A–C is incomparable; partly because of the change in particle size (different sampling conditions for whole and ground pellets) which influences the algorithm evaluation of the surface ROI, partly because of the utilized wavelength that is mainly representative when astaxanthin is present. This also explains the insignificant correlation for the standard curve for the whole pellets at the end of the storage period where the astaxanthin concentration is very low.

The outcome of the chemical analyses (HPLC) at the start and end of the storage period signifies once again that astaxanthin is present *in* the samples even though only very low levels are measured *on* the sample surfaces, and that the degradation of the astaxanthin during storage mainly seems to be linked to the light. In the groups with initial low levels of added astaxanthin, K2-K4, the degradation seems to level out when 20% of the initial concentration is left. In all, this implies that astaxanthin has self-protective properties. Torrissen (1984) describes two mechanisms that are likely to be involved in the self-protection observed in the present study: As a carotenoid, astaxanthin is able to protect against photosensitized oxidation by either quenching singlet excited oxygen or acting as a pigmentation filter.

During the storage period, the fish feed pellets started to stick to the lid of the Petri dishes, and the sticky behaviour was deferred with increased astaxanthin concentration. The same deferral is seen for the minimum relative weight of the samples, constituting a possible correlation between the processes causing the sticky pellets and the weight loss. This reflection originates from the physical observations, and there has not been accounted for supplementary sample material to perform elucidating analyses of the underlying reactions. However, BioMar reports that fish meal starts to stick and cake at the end of its shelf life. Similar stickiness is reported by Schaich (2014) in a study evaluating peanut butter during storage, and sticky behaviour is suggested to originate from oxidation of lipids and proteins.

5. Conclusions

Following seven groups of fish feed pellets with different initial concentrations of astaxanthin, it was shown that the degradation of astaxanthin primarily takes place on the light-exposed parts of the surface of the fish feed pellets. The controlled illumination during storage, equivalent to the daylight intensity present at extensive clouding (Jobling, 2010), simulated mild and realistic conditions present at many fish farms during a time span within the date for final use (personal communication: Anders Falk Andreasen, BioFarm at BioMar, Denmark, 2015). As oxygen is known to degrade astaxanthin as well, it is very likely that evaluation of oxygen influence would lead to similar conclusions. Even though the concentration of astaxanthin on the surface is reduced to the same level among the different groups of pellets, 20–30% of the initial concentration is kept and preserved inside the pellets at the end of the storage period. Independent of the initial astaxanthin

concentration, there is a constant delay before the degradation of astaxanthin starts. As the degradation is most noticeable at the surface, it is reasonable to assume that the delay of degradation can be extended be coating and protecting only the surface of the pellets with another antioxidant, and in all circumstances aware unnecessary light exposure. Alternatively, astaxanthin should preferably be introduced specifically to the core structure of the pellet rather than on or close to the surface. Consequently, larger pellets with higher core volume/surface area ratio have more optimal geometrical conditions to protect astaxanthin compared to small pellets where its localization in the centre core is of even higher importance. Furthermore, the results demonstrated a concentration dependent antioxidative effect of astaxanthin delaying the pigmentation degradation attributing self-protective properties to astaxanthin when especially used in high concentrations. As a result of these antioxidative and self-protective properties, astaxanthin should besides the well-known function for flesh pigmentation also be considered for the ability to prevent oxidation and extend the shelf life of fish feed pellets.

Acknowledgements

For technical support, we would like to thank Heidi Olander Petersen and Bjørn Skovlund Dissing. We would also like to thank Aberham Hailu Feyissa for critical reading of the manuscript.

This project is funded by the Danish Ministry of Food, Agriculture and Fisheries (J-34009-12-0469) and Innovation Fund Denmark (4135-00036).

References

Anarjan, N., Tan, C.P., 2013. Effects of storage temperature, atmosphere and light on chemical stability of astaxanthin nanodispersions. J. Am. Oil Chem. Soc. 90 (8), 1223–1227.

Armenta, R.E., Guerrero-Legarreta, I., 2009. Stability studies on astaxanthin extracted from fermented shrimp byproducts. J. Agric. Food Chem. 57 (14), 6095–6100.

Boon, C.S., McClements, D.J., Weiss, J., Decker, E.A., 2010. Factors influencing the chemical stability of carotenoids in foods. Crit. Rev. Food Sci. Nutr. 50 (6), 515–532.

Choubert, G., Storebakken, T., 1989. Dose response to astaxanthin and canthaxanthin pigmentation of rainbow trout fed various dietary carotenoid concentrations. Aquaculture 81 (1), 69–77.

Franco-Zavaleta, M.E., Jiménez-Pichardo, R., Tomasini-Campocosio, A., Guerrero-Legarreta, I., 2010. Astaxanthin extraction from shrimp wastes and its stability in 2 model systems. J. Food Sci. 75 (5), C394–C399.

Hencken, H., Estermann, R., 1991. Pigmenting agents in fish Feed—Current knowledge and practice. Aquac. Ind. Dev. Rep., 34–51 (ISSN 1183-0352).

Hernández, A., García, B.G., Jordán, M.J., Hernández, M.D., 2014. Natural antioxidants in extruded fish feed: protection at different storage temperatures. Anim. Feed Sci. Technol. 195, 112–119.

Jobling, M., 2010. Fish culture: the rearing environment. I. In: Le François, N.R. (Ed.), Finfish Aquaculture Diversification. CABI Publishing, Oxfordshire, UK, pp. 33–60 (ISBN-13: 9781845934941).

Johnson, E.A., An, G.-H., 1991. Astaxanthin from microbial sources. Crit. Rev. Biotechnol. 11 (4), 297–326.

Kitts, D.D., Tomiuk, S., 2013. Studies on mitigating lipid oxidation reactions in a value-added dairy product using a standardized cranberry extract. Agriculture 3 (2), 236–252.

Ljungqvist, M.G., Nielsen, O.H.A., Frosch, S., Nielsen, M.E., Clemmensen, L.H., Ersbøll, B.K., 2014. Hyperspectral imaging based on diffused laser light for prediction of astaxanthin coating concentration. Mach. Vis. Appl. 25 (2), 327–343.

Ljungqvist, M., 2012. Multivariate Image Analysis for Quality Inspection in Fish Feed Production. Technical University of Denmark, Lyngby, Denmark.

V. Marleen, 2007. Coated animal feed with active compounds, Eur. Pat. Appl. EP1825762A1.

Schaich, K.M., 2002. Free radical generation during extrusion: a critical contributor to texturization. ACS Symp. Ser. 807, 35–48.

Schaich, K.M., 2014. One Size Does Not Fit All: Liid Co-oxidation of Roteins.

Sigurgisladottir, S., Parrish, C.C., Lall, S.P., Ackman, R.G., 1994. Effects of feeding natural tocopherols and astaxanthin on Atlantic salmon (*Salmo salar*) fillet quality. Food Res. Int. 27 (1), 23–32.

Torrissen, O.J., Christiansen, R., 1995. Requirements for carotenoids in fish diets. J. Appl. Ichthyol. 11 (3–4), 225–230.

Torrissen, O.J., Naevdal, G., 1988. Pigmentation of salmonids—variation in flesh carotenoids of atlantic salmon. Aquaculture 68 (4), 305–310.

Torrissen, O.J., 1984. Pigmentation of salmonids −Effect of carotenoids in eggs and start-feeding diet on survival and growth rate. Aquaculture 43 (1–3), 185–193.

Torrissen, O.J., 1985. Pigmentation of salmonids: factors affecting carotenoid deposition in rainbow trout (*Salmo gairdneri*). Aquaculture 46 (2), 133–142.


Brande